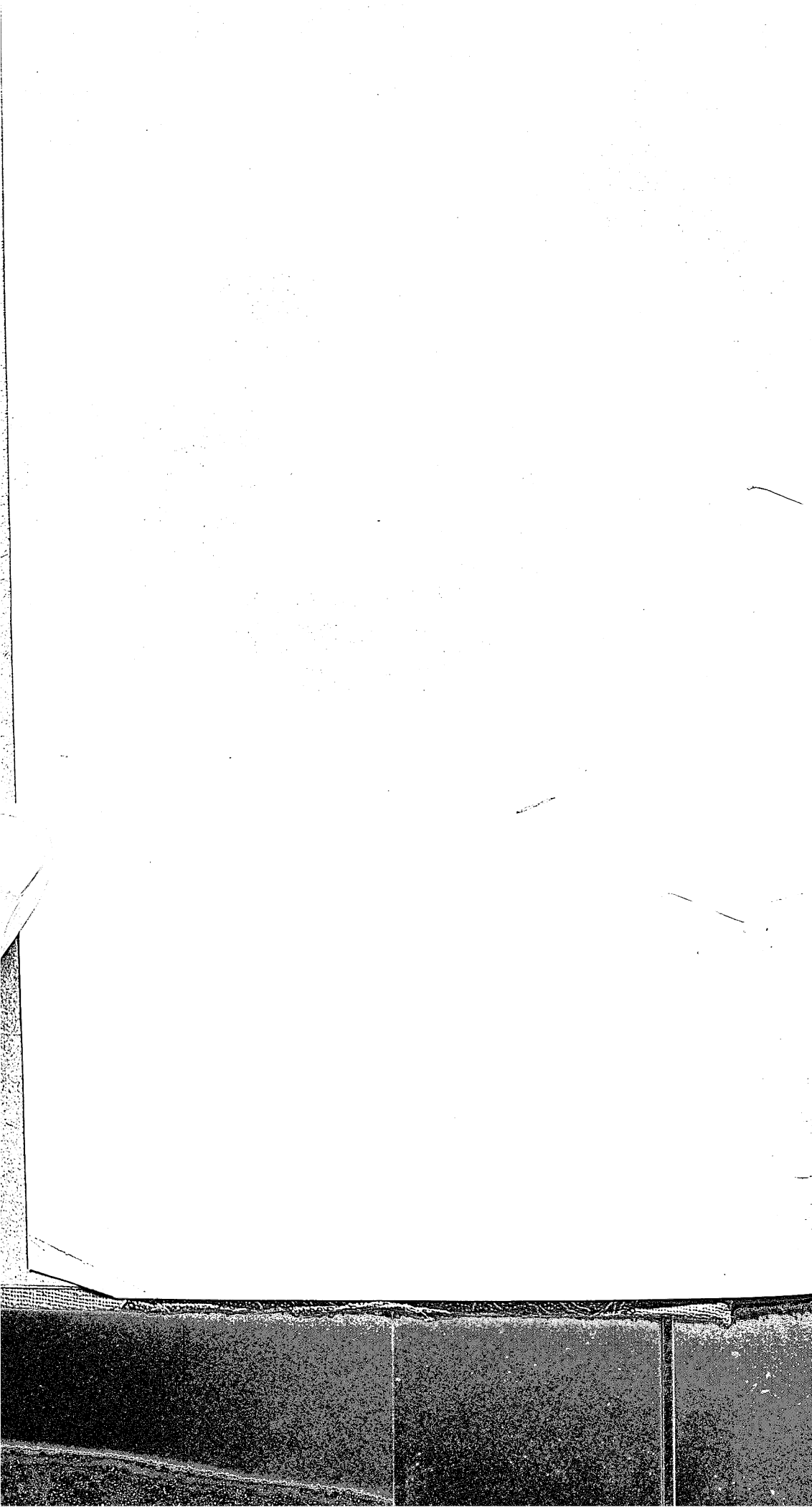
JOURNAL
OF
DAIRY SCIENCE

VOLUME XLI
JANUARY–DECEMBER 1958

1958
THE AMERICAN DAIRY SCIENCE ASSOCIATION



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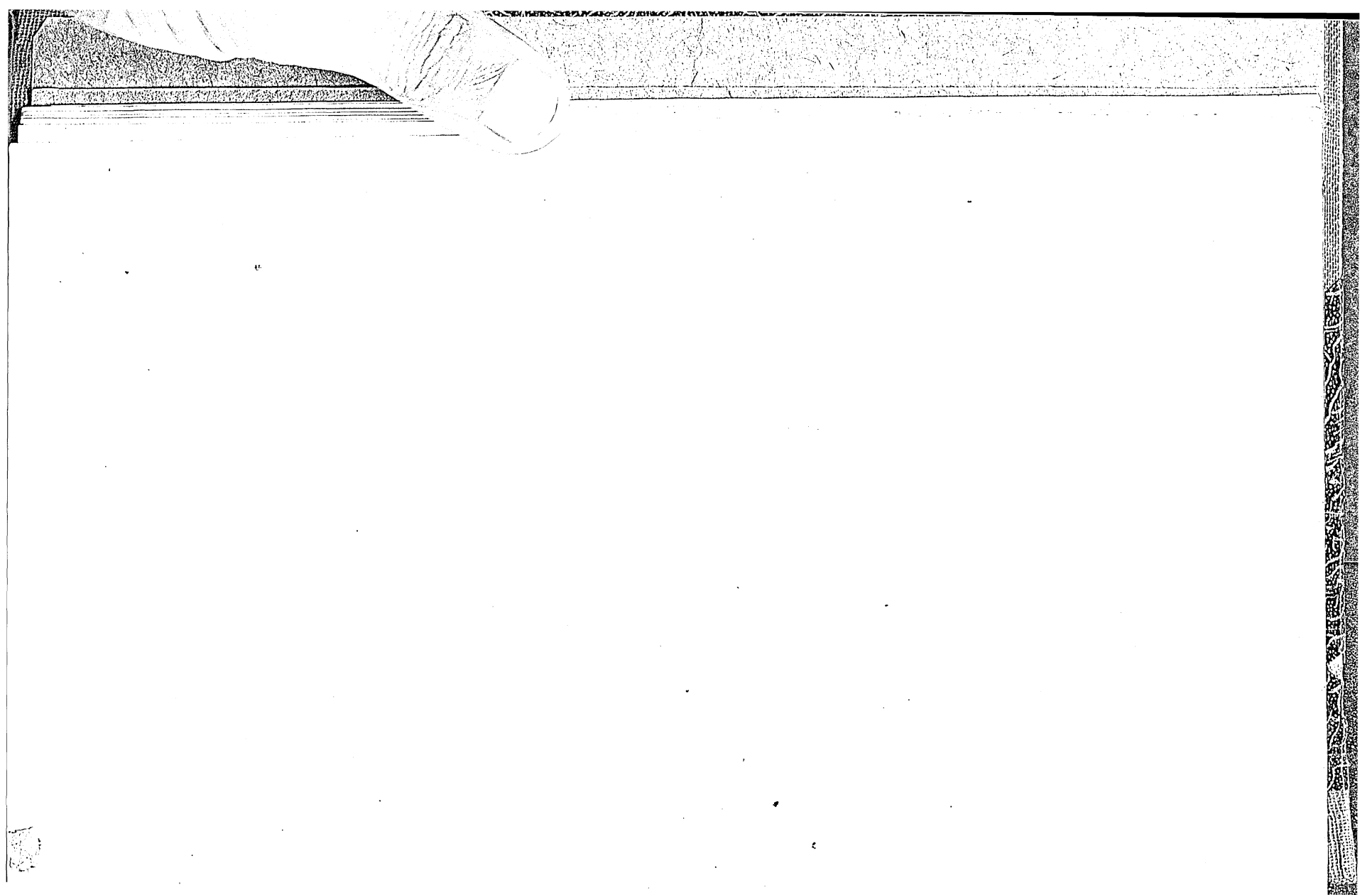
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A COMPARISON OF SOME OF THE CHEMICAL AND PHYSICAL PROPERTIES OF γ-CASEIN AND IMMUNE GLOBULINS OF MILK[1]

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SUMMARY

To identify the slowest component in the electrophoretic pattern of skimmilk at pH 8.7 and to investigate further the chemical and physical properties of γ-casein and the immune globulins, these proteins were isolated from milk and colostrum. Phosphorus analyses indicate a distinct difference between γ-casein and immune globulins. In veronal buffer at pH 8.7, $\mu = 0.1$, the electrophoretic mobilities of γ-casein and pseudoglobulin are the same. In glycine hydrochloride buffer pH 2.3, $\mu = 0.1$, the three proteins have different electrophoretic mobilities, but a mixture of γ-casein-euglobulin interacts, the extent depending on the time of storage at 4° C. While γ-casein is electrophoretically homogeneous in glycine hydrochloride buffer at pH 2.3 and 3.25 and $\mu = 0.1$, it is heterogeneous in comparable sodium lactate buffers, due to interactions with the lactate ion. All three proteins contribute to the area of the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.7. They are heterogeneous in the ultracentrifuge. The molecular weight calculated for the major components of γ-casein varied with pH; whereas, the frictional ratios were the same in veronal and glycine hydrochloride buffers but decreased in sodium lactate buffer. The molecular weights of euglobulin and pseudoglobulin were not pH-dependent, but their frictional ratios increased with decreasing pH values.

Recently, the Protein Committee *(11)* of the American Dairy Science Association has clarified the current nomenclature for the milk proteins. They have recognized, on the basis of the work of McMeekin *(15)*, that the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.4–8.7 and 0.1 ionic strength is the γ-casein. However, Heyndrickx and DeVleeschauwer *(6, 7)* neither recognized nor reported the presence of γ-casein, and they have attributed a globulin character to the same component, both in colostrum and in milk. Comparison of some of the properties of γ-casein and immune globulins available in the literature *(4, 8, 21, 22)* shows that these proteins have approximately the same isoelectric point, electrophoretic mobility at pH 8.7, nitrogen, sulfur, lysine, and valine contents. In order to clearly identify the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.7, and further to compare these proteins, γ-casein and the immune globulins were isolated from milk and colostrum, respectively, and some of their chemical and physical properties were studied.

Received for publication August 23, 1957.

[1] Taken from the thesis of G. K. Murthy, presented in June, 1956, to the University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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MATERIALS AND METHODS

γ-Casein. Casein was prepared according to Cherbuliez and Baudet *(2)* from selected Holstein cows' milk and fractionated to obtain γ-casein, by the method described by Hipp *et al. (8)*.

Immune globulins. Euglobulin and pseudoglobulin were prepared from colostrum, first milking after parturition, by the method described by Smith *(21)* for their isolation from bovine whey.

Phosphorus determination. Phosphorus contents of the proteins were determined using Verma's *(24)* technique for digesting the samples, and Fiske and Subbarow's *(3)* method for color development. The blue color developed was measured in a Coleman Model 11 Spectrophotometer at 660 mμ.

Electrophoretic analyses. Electrophoretic analyses of the isolated proteins were performed at ice bath temperatures in sodium veronal (pH 8.7,$\mu = 0.1$), glycine hydrochloride (pH 2.3, $\mu = 0.1$), and sodium lactate (pH 2.3, $\mu = 0.1$) buffers. One per cent protein suspensions were prepared either singly or in mixtures, in the ratio of 1:1. Since the electrophoretic pattern of γ-casein-euglobulin mixture obtained at pH 2.3 was unexpected, the analysis was repeated after 24 hr. of storage of the mixture at 4° C.

Since the electrophoretic patterns of γ-casein in lactate buffer at pH 2.3 were heterogeneous, possibly due to the low buffer capacity of this buffer at this pH, a special comparison was made of the electrophoretic pattern of γ-casein in glycine and lactate buffers at pH 3.25 and $\mu = 0.1$, where the buffer capacities of the two systems were the same.

In order to confirm the identity of the slow-moving component of skimmilk at pH 8.7, γ-casein, euglobulin, and pseudoglobulin each were added, at 0.1% concentration, to a portion of skimmilk diluted 1:2.5 with sodium veronal buffer, assuming the protein concentration of the diluted skimmilk to be 1.0%. These systems were then electrophoretically analyzed in the usual manner.

Diffusion analyses. Diffusion analyses of most of the same protein suspensions prepared for electrophoresis were performed at 2° C. in a Tiselius electrophoresis apparatus. The initial boundaries were created according to the method described by Lundgren and Ward *(14)*, and the diffusion constant was calculated by the area and height method. The values were corrected to standard conditions in the usual manner.

Viscosity. The viscosity measurements were made with the Höeppler viscosimeter *(10)*. In order to avoid the use of instrument constants which would change with temperature, the viscosity of the buffers was calculated by comparison with water over the temperature range of from 0 to 10° C. The viscosity of water at the various temperatures was obtained from Lange *(12)*.

Density. The densities of the buffers at the various temperatures were determined with the Westphal balance.

Sedimentation analyses. Sedimentation analyses were carried out at from 4 to 6° C. in a Spinco Model E Ultracentrifuge, with 1.0 and 0.5% protein suspensions in sodium veronal and glycine hydrochloride buffers. For compari-

son purposes, γ-casein was analyzed at 0.5% protein in sodium lactate and glycine hydrochloride buffers of pH 2.3 and pH 3.25, and $\mu = 0.1$. The observed sedimentation constants were corrected to standard conditions in the usual manner. The partial specific volumes were obtained from the literature *(15, 21)*. The $S_{20,w}$ values were extrapolated to zero protein concentration and the extrapolated values were used in the calculation of apparent molecular weight.

RESULTS AND DISCUSSION

The phosphorus content of γ-casein (0.107%) is in close agreement with Hipp's value—0.11% *(8)*. Analyses of the immune globulins support the findings of Smith *(20, 21)*, in that they do not indicate the presence of any phosphorus; therefore, the globulins in this respect show a marked difference from γ-casein.

Electrophoretic analyses. Results of the electrophoretic analyses are presented (Table 1). The isolated γ-casein contained from 5 to 8% of a slow-moving component which might be the δ-casein, as suggested by Cherbuliez and Baudet *(2)*. In veronal buffer, the mobility of γ-casein conforms with Hipp's *(8)* value, -2.01×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}. The mobility of euglobulin is approximately the same as that observed by Smith *(22)*, -1.8×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}; whereas, that of pseudoglobulin is different from his value of -2.2×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}. The γ-casein and pseudoglobulin have practically the same mobility

TABLE 1

Electrophoretic properties of γ-casein and immune globulins

Protein	Descending		Ascending	
	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)
	Veronal buffer, pH 8.7, $\mu = 0.1$, 1% protein			
γ-Casein	100	−2.03	100	−2.32
Euglobulin	100	−1.76	100	−1.81
Pseudoglobulin	100	−2.04	100	−2.13
γ-Casein + euglobulin (1:1)	53.3	−1.74	51.8	−1.85
	46.7	−2.03	48.2	−2.32
γ-Casein + pseudoglobulin (1:1)	100	−2.00	100	−2.30
	Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$, 1% protein			
γ-Casein	100	3.23	100	4.33
Euglobulin	100	5.28	100	6.12
Pseudoglobulin	100	5.17	100	5.99
γ-Casein + euglobulin (1:1)	13.9	2.14	53.6	4.41
44-hr. dialysis	51.4	3.57	46.4	5.97
	34.7	5.19		
γ-Casein + euglobulin (1:1)	27.1	3.05	56.4	4.54
44-hr. dialysis +	45.0	4.09	43.6	5.97
24-hr. storage	27.8	5.19		
γ-Casein + pseudoglobulin (1:1)	50	3.39	49.1	4.32
	50	5.12	50.9	6.01

and in mixtures move with a single maximum. In mixtures, γ-casein and euglobulin move independently with the same mobility as they possess in the isolated systems and, therefore, indicate no complex formation between them.

In glycine hydrochloride buffer, the patterns of γ-casein and immune globulins are sharper than in veronal buffer. While the γ-casein and pseudoglobulin move independently in mixtures showing no complex formation between them, the γ-casein-euglobulin mixture resulted in three components on the descending side (Figure 1), the faster component having the mobility of euglobulin and the

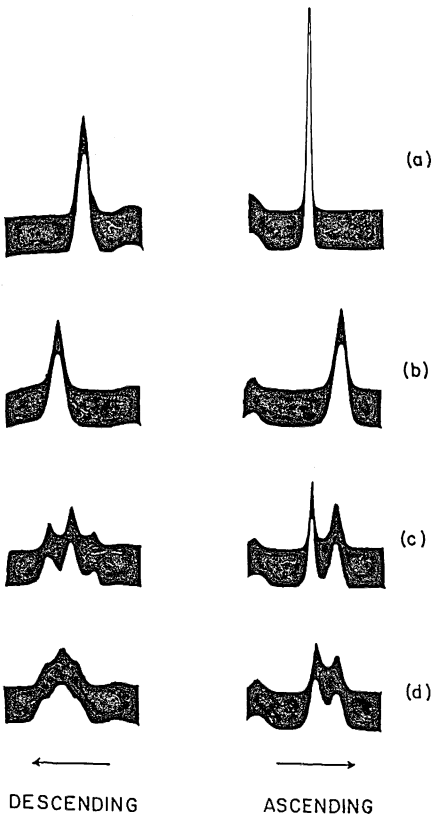


FIG. 1. Electrophoretic patterns obtained in a glycine-HCl buffer (pH 2.3 ionic strength of 0.1) with a protein concentration of 1.0%, after electrophoresis for 4,500 sec. *(a)* γ-casein at field strength of 9.35 V/cm; *(b)* euglobulin at field strength of 9.16 V/cm; *(c)* γ-casein-euglobulin (44-hr. dialysis) at field strength of 9.09 V/cm; *(d)* γ-casein-euglobulin (44-hr. dialysis, 24 hr. of storage) at field strength of 9.09 V/cm.

other two components differing in mobility, one faster and the other slower than γ-casein. On the ascending side, only two components are observed, with the faster component having the same mobility as euglobulin and the slower component moving faster than γ-casein. To confirm this unexpected result, the electrophoretic analysis was repeated after 24 hr. of storage at 4° C., and a similar result was obtained; however, the relative areas of the various components on the

descending side were found to have changed (Figure 1), along with their mobilities, except for the mobility of the fastest component, euglobulin. These results indicate the possibility of a complex formation similar to that observed by Chargaff *et al. (1),* in their electrophoretic studies of serum albumin-heparin mixtures, and by Longsworth and McInnes *(13),* with mixtures of ovomucoid and nucleic acid. Since there was a change in the electrophoretic pattern with time of storage, the rate of attainment of equilibrium is very slow when compared to electrophoretic separation. However, the association observed cannot be explained from the interaction types proposed by Longsworth, since, in this case, the velocity constant for the forward reaction, k_1, and for the reverse reaction, k_2, are both small, but k_1 is greater than k_2.

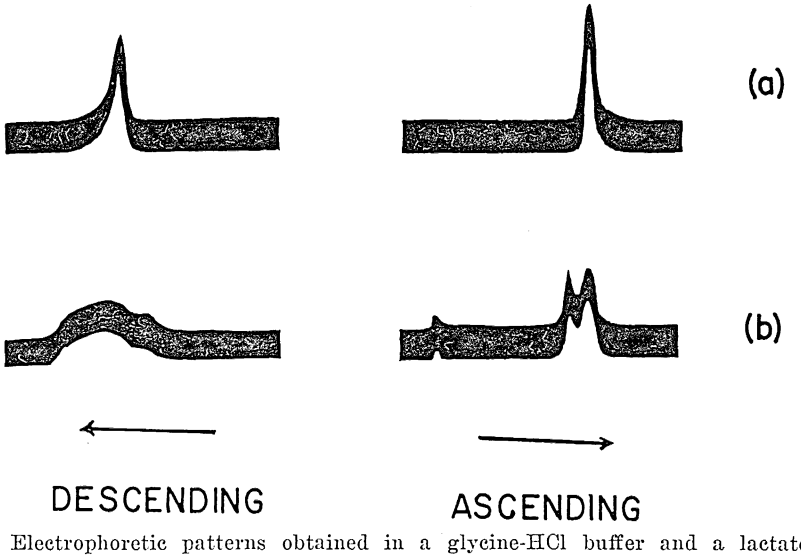


FIG. 2. Electrophoretic patterns obtained in a glycine-HCl buffer and a lactate buffer (pH 3.25 ionic strength of 0.1) with a protein concentration of 0.5%, after electrophoresis for 5,400 sec. *(a)* γ-casein in glycine-HCl buffer at field strength of 10.69 V/cm; *(b)* γ-casein in lactate buffer at field strength of 12.62 V/cm.

γ-Casein is electrophoretically homogeneous in glycine hydrochloride buffer of pH 2.3 and 3.25, and $\mu = 0.1$, and it is heterogeneous in the comparable sodium lactate buffers (Figure 2). This behavior of γ-casein in lactate buffer is similar to that observed by Hipp *et al. (8).* The patterns obtained at pH 3.25 show that this heterogeneity is not due to a low buffer capacity but to an interaction with the buffer ion.

The electrophoretic properties (Table 2) of the isolated γ-casein, euglobulin, and pseudoglobulin, upon their addition to skimmilk, show that these proteins, irrespective of their mobilities in the isolated system, behave similarly. In skimmilk, they all appear as the slow-moving component, with approximately the same mobility of -2.01×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}.

Diffusion. The observed diffusion constants, $D_{2,b}$ (Table 3), in veronal buffer for the immune globulins, are approximately the same as those reported

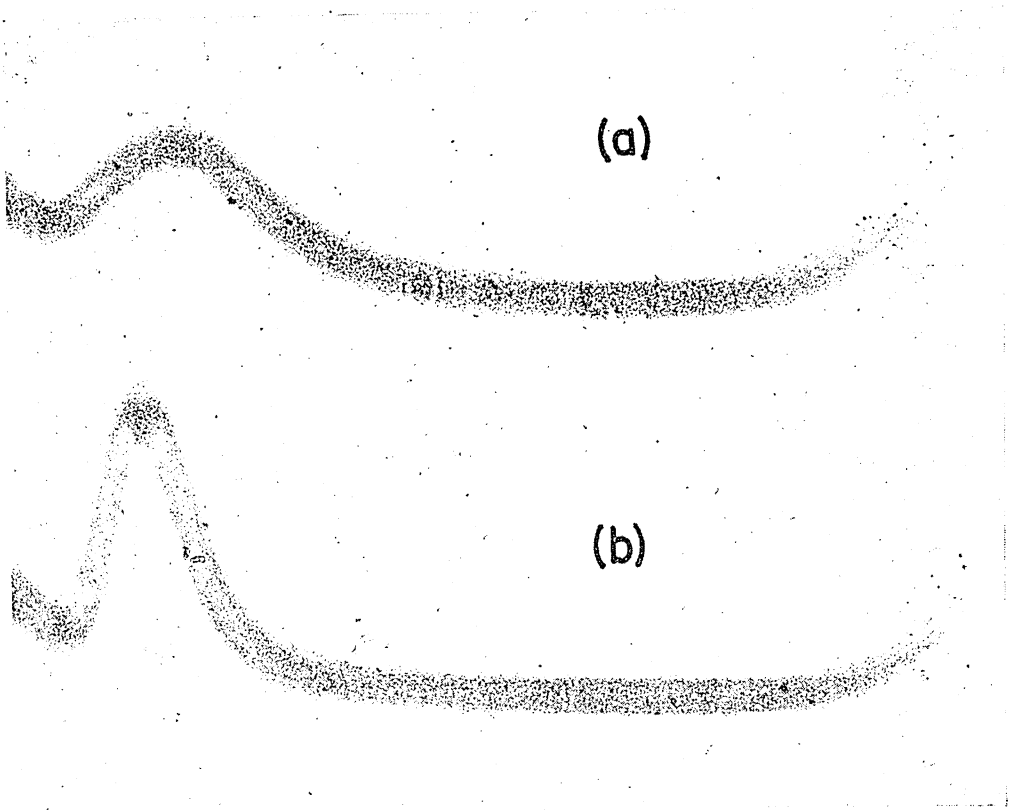


FIG. 3. Sedimentation pattern obtained in a glycine-HCl buffer and lactate buffer (pH 3.25, ionic strength of 0.1) with a protein concentration of 0.5% at 12.5° C. *(a)* γ-casein in glycine-HCl buffer, *(b)* γ-casein in lactate buffer.

by Smith *(21)*. However, the diffusion constants calculated at standard conditions are lower. This may be owing to the differences in the measured viscosities of the buffer and the methods employed in their determination. The diffusion constant of γ-casein is considerably higher than that of the immune globulins. The γ-casein-pseudoglobulin mixture diffuses at a slightly faster rate than the average of their diffusion constants. The relative areas under the diffusion curves of the mixtures, γ-casein, and pseudoglobulin are significantly different; therefore, the rate of change of index of refraction with concentration of the individual components must be different in mixtures than it is in the individual systems. Hence, the slightly faster than average diffusion rate of the mixture may not indicate any interaction between them, since Gosting *et al. (5)* show that the diffusion constant of a mixture in which there is no interaction need not be the weighted average of the diffusion constants of the components, provided the rates of change of index of refraction with concentration for the individual components are different. In glycine hydrochloride buffer, the diffusion constants of all three proteins are lower than in veronal buffer, with the euglobulin and pseudoglobulin diffusing at the same rate. The diffusion constant of the γ-casein and euglobulin mixture is as expected for a 50:50

TABLE 2

Electrophoretic properties of isolated γ-casein and immune globulins upon their addition to skimmilk [a]

Sample	α-Casein		β-Lactoglobulin		β-Casein		Component 4	
	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)
				Descending				
Skimmilk	69.3	−5.61	5.2	−4.59	24.4	−3.17	1.0	−1.98
Skimmilk + γ-casein	64.6	−5.51	6.1	−4.59	21.3	−3.17	8.0	−2.02
Skimmilk + euglobulin	65.0	−5.61	6.4	−4.59	21.5	−3.17	7.1	−2.02
Skimmilk + pseudoglobulin	64.5	−5.40	6.1	−4.50	21.6	−3.05	7.6	−2.05
				Ascending				
Skimmilk	61.8	−6.43	7.4	−5.56	29.2	−4.00	1.6	−2.80
Skimmilk + γ-casein	57.9	−6.34	7.1	−5.42	26.0	−3.95	9.0	−2.78
Skimmilk + euglobulin	58.0	−6.43	7.3	−5.42	25.4	−3.95	9.2	−2.66
Skimmilk + pseudoglobulin	57.2	−6.34	7.9	−5.42	25.6	−3.91	9.3	−2.66

[a] Electrophoresis was performed at ice-bath temperatures in veronal buffer, pH 8.7, $\mu = 0.1$.

TABLE 3

Diffusion constants of γ-casein and immune globulins

	Diffusion constant	
	$D_{2,b}$	$D_{20,w}$
	(cm.² sec.⁻¹ × 10⁷)	
	Veronal buffer, pH 8.7, $\mu = 0.1$, 1%protein	
γ-Casein	2.71 ± 0.06	4.82
Euglobulin	1.63 ± 0.04	2.91
Pseudoglobulin	1.82 ± 0.04	3.23
γ-Casein + pseudoglobulin (1:1)	2.40 ± 0.06	4.26
	Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$, 1%protein	
γ-Casein	1.13 ± 0.10	1.87
Euglobulin	1.59 ± 0.04	2.60
Pseudoglobulin	1.59 ± 0.04	2.60
γ-Casein + euglobulin (1:1)	1.30 ± 0.05	2.16
	Lactate buffer, pH 2.3, $\mu = 0.1$, 1%protein	
γ-Casein	1.13 ± 0.03	2.18

mixture, assuming no interaction; however, the observed value must be the average of the various species of γ-casein and euglobulin present. In sodium lactate buffer (pH 2.3), γ-casein diffuses slightly faster than in glycine hydrochloride buffer (pH 2.3).

Sedimentation. As expected, the rate of sedimentation of γ-casein, euglobulin, and pseudoglobulin are concentration-dependent, increasing with decreasing protein concentration. In veronal buffer, γ-casein has a low $[S_{20,w}]_{c=o}$ value of 1.55 Svedberg units, and sediments with a single, although broad, maximum. Prolongation of sedimentation time during the analysis did not reveal any additional maxima, but the spreading of the peak is greater than predicted by diffusion. However, in glycine hydrochloride buffer, γ-casein possesses at least three components sedimenting close to one another, the major component, 67%, having a $[S_{20,w}]_{c=o}$ value of 10.30 Svedberg units. In lactate buffer (pH 2.3, and 3.25, $\mu = 0.01$) it is more homogeneous. McMeekin and Peterson (16) observed that γ-casein is heterogeneous in the ultracentrifuge (the rates of sedimentation of the components depending upon the temperature of analysis) and that the system behaves reversibly. It appears from this study that the sedimentation of γ-casein depends also on the pH and the type of buffer used. The euglobulin and pseudoglobulin are heterogeneous both in veronal and glycine hydrochloride buffers. The euglobulin yielded three components with the major component, from 86 to 87% of the total area; whereas, the pseudoglobulin yielded two components with the major component, from 84 to 89.3% of the total area. These results are in good agreement with the sedimentation constants obtained by Smith and Brown *(23)* in 0.15 *M* NaCl at room temperature.

Molecular weights. Although the heterogeneity of these proteins is readily apparent, it is interesting to calculate the apparent molecular weights of

TABLE 4

Sedimentation constants of γ-casein and immune globulins

Protein	Sedimentation constant ($S_{20,w}$)		
	Protein		
	1.0%	0.5%	0.0%
	Veronal buffer, pH 8.7, $\mu = 0.1$		
γ-Casein	1.41 (100%)	1.49	1.55
Euglobulin	7.93 (87.0%)	8.36	8.77
	11.85 (10.0%)	11.81	11.7
	22.98 (3.0%)		22.0
Pseudoglobulin	7.69 (89.3%)	7.91	8.07
	10.7 (10.7%)	10.94	11.17
	Glycerine hydrochloride buffer, pH 2.3, $\mu = 0.1$		
γ-Casein	— (15.0%)	—	—
	9.72 (67.0%)	10.03	10.30
	— (18.0%)	—	—
Euglobulin	6.91 (86.0%)	7.33	7.75
	10.51 (11.0%)	—	—
	— (3.0%)	—	—
Pseudoglobulin	7.04 (84.0%)	7.10	7.18
	10.21 (12.0%)	10.30	10.39
	— (4.0%)	—	—
	Glycerine hydrochloride buffer, pH 3.25, $\mu = 0.1$		
γ-Casein		—	
		17.39	
	Lactate buffer, pH 2.3, $\mu = 0.1$		
γ-Casein		14.54 (100%)	
	Lactate buffer, pH 3.25, $\mu = 0.1$		
γ-Casein		11.48 (100%)	

the major components of euglobulin and pseudoglobulin from sedimentation and diffusion constants $[S_{20,w}]_{c=0}$ and $[D_{20,w}]_{c=1.0\%}$ (Table 3). The molecular weights of each are approximately the same, either in veronal or glycine hydrochloride buffer, with an average value of 252,000 and 289,000, respectively. Smith *(22)* reported the molecular weights of these immune globulins (milk and colostrum) to be in the neighborhood of from 160,000 to 190,000. This discrepancy may be owing to the methods of selection of the values for diffusion and sedimentation constants to be used in Svedberg's formula. He assumed a value for the diffusion constant greater than 3.9×10^{-7} cm.^{2} sec^{-1}, although his values for the immune globulin varied between 3.24 and 3.52×10^{-7} cm.^{2} sec.$^{-1}$ for bovine whey and 3.86×10^{-7} cm.^{2} sec.$^{-1}$ for colostrum pseudoglobulin, and he gave a value of 7.0 Svedberg units for their sedimentation constant. It is not clear whether or not the sedimentation constant used was obtained by extrapolation to zero protein concentration.

The molecular weights of γ-casein (Table 5) show large differences as a result of environment, indicating association of the protein molecule at low pH values.

Frictional ratios. As in the case of the calculation of molecular weights, the heterogeneity of the protein raises some question as to the validity of calculated frictional ratios. However, the molar frictional ratios (f/f_o), for

TABLE 5

Molecular weight of major component of γ-casein and immune globulins

Protein	Molecular weight		
	Veronal buffer (pH 8.7, $\mu = 0.1$)	Glycine HCl buffer (pH 2.3, $\mu = 0.1$)	Lactate buffer (pH 2.3, $\mu = 0.1$)
γ-Casein	30,650	537,000	659,000
Euglobulin	291,000	287,000	
Pseudoglobulin	243,000	262,000	

the immune globulins (Table 6) are greater than unity and, therefore, indicate that these protein molecules are either hydrated or asymmetrical. However, when the values for (f/f_o) are expressed as products of hydration (f/f_e) and asymmetry (f_e/f_o), assuming reasonable values for hydration, the results can not be explained on the basis of hydration alone. The axial ratios calculated using Perrin's *(18)* equation are higher at pH 2.3 than at pH 8.7, although the molecular weights are approximately the same at both the pH levels. It is possible to explain these observations on the basis of changes in asymmetry with pH. Polson *(19)* and Neurath *(17)* have calculated the minimum value for the radius of minor axis of several proteins to be from 8 to 9 Å. At pH 8.7, the calculated radius of the minor axis at constant hydration (0.2 g. bound water per gram of protein) can be approximated if the cross section is assumed to consist of four protein units of radius 8.5 Å; whereas, at pH 2.3, it can be approximated with three protein units in the cross section of the molecule. On the other hand, at a constant value for the axial ratios, the changes in the frictional ratios can be explained on the basis of a change in hydration only in the case of euglobulin. For pseudoglobulin, it would be necessary to assume unreasonable values for hydration.

For γ-casein, the observed axial ratios calculated for an elongated ellipsoid differ markedly from those reported by Hipp *et al. (9)*, who obtained values

TABLE 6

Frictional ratios and dimenions of γ-casein and immune globulins

Protein		Hydrated (0.2 g. H_2O/g protein)			Hydrated (0.8 g. H_2O/g protein)		
			Axial ratio	b		Axial ratio	b
	f/f_o	f_e/f_o	(a/b)	(Å)	f_e/f_o	(a/b)	(Å)
			Veronal buffer, pH 8.7, $\mu = 0.1$				
γ-Casein	2.080	1.944	19.4	8.5	1.687	12.9	9.12
Euglobulin	1.648	1.526	9.6	20.9	1.308	5.9	24.40
Pseudoglobulin	1.587	1.469	8.6	20.3	1.260	5.5	23.60
			Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$				
γ-Casein	2.100	1.944	19.4	20.2	1.667	12.4	23.20
Euglobulin	1.846	1.709	13.2	18.6	1.465	8.5	21.50
Pseudoglobulin	1.895	1.754	14.3	17.5	1.504	9.2	20.40
			Lactate buffer, pH 2.3, $\mu = 0.1$				
γ-Casein	1.710	1.600	11.0	26.0	1.357	6.7	30.70

of 9.3 for the elongated and 16.2 for the prolate molecule, based upon the intrinsic viscosity measurements in NaOH–NaCl system of pH 7.2 and $\mu = 0.05$. The lack of agreement is undoubtedly due to the differences in solvent systems and the methods of measurement. No difference in the axial ratios in veronal and glycine hydrochloride buffers is observed. The observed value of 8.5 Å for the radius of the minor axis of γ-casein is in close agreement with the minimum values of from 8 to 9 Å calculated by Polson *(19)* and Neurath *(17)*. However, the molecular weight in glycine hydrochloride buffer is approximately 18 times larger than that in veronal buffer. To account for the increase in molecular weight without appreciable change in the axial ratios, we can postulate an aggregation of 18 of the molecular species present at pH 8.7, with a maximum cross section of four of them. Such a molecule at 0.2 g. of bound water per gram of protein would have a radius of 19.6 Å, compared with the calculated radius of 20.2 Å.

The molecular weight in lactate buffer is approximately 22 times larger than in veronal buffer, with appreciable changes in frictional ratios. These differences can be explained by assuming an aggregation of 22 of the molecular species present at pH 8.7 and at 0.8 g. of bound water per gram of protein, with a maximum cross section of eight of them. Such a molecule will have a radius of 30.0 Å, compared with the calculated radius of 30.7 Å.

In conclusion, the above observations show: that γ-casein and immune globulins, although different proteins, are all components of the slow-moving peak in the electrophoretic pattern of skimmilk at pH 8.7; the molecular weights of the immune globulins are different from those reported by Smith, and the molecular weight of γ-casein is a function of pH and buffer ion as well as temperature; whereas, the hydration and/or asymmetry of all the proteins studied are pH-dependent.

ACKNOWLEDGMENT

We wish to thank Dr. T. L. McMeekin, Eastern Utilization Research Branch, USDA, for generously supplementing our supply of γ-casein; Dr. Bruce Larson, Department of Dairy Science, University of Illinois, for selecting cows' milk containing high γ-casein content and for supplying colostrum, and Dr. B. R. Ray, Department of Chemistry, University of Illinois for making the ultracentrifuge available.

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SEPARATION, CONCENTRATION, AND PROPERTIES OF ALPHA-AMYLASE FROM COWS' MILK[1]

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SUMMARY

The presence in milk of α-amylase was confirmed. Analyses for the enzyme in milk and fractions thereof were made by measuring the rate of decrease in viscosity of starch paste with Ostwald viscosimeters. Analysis of milk and that dialyzed with various added salts revealed that the enzyme requires both calcium and chloride for activity. When milk proteins were fractionated, much of the α-amylase activity was concentrated into the lactoglobulin fraction precipitated from whey at 43% saturation with $(NH_4)_2SO_4$. The enzyme was further concentrated by dissolving the lactoglobulin in a solvent consisting of ethylene glycol, ethanol, and water in the ratio of 15:29:56 by volume, adsorbing it upon rice starch and eluting with saturated $CaSO_4$ solution. A preparation with 30 times the specific activity of lactoglobulin and containing 20% of the total activity of the lactoglobulin from which it was prepared, had starch liquefying, dextrinizing, and saccharifying properties. The optimum pH for the enzyme was 7.4 at 34° C. The energy of activation of the catalyzed reaction, measured by starch liquefaction, was 8,500 calories per mole. The enzyme was progressively inactivated by 30-min. heating between 45 and 52° C. at pH 6.4. A plot of velocity vs. velocity/substrate concentration was linear, indicating a single enzyme entity.

Alpha-amylases hydrolyze starch and glycogen by splitting central α-1-4-D-glucosidic linkages, causing rapid liquefaction and slower dextrinization and saccharification. They are found in plants, microorganisms, and in the pancreas, blood, urine, and saliva of animals *(8)*. They usually require either calcium or chloride ions. On the other hand, β-amylases cause rapid saccharification of starch with slow liquefaction, by splitting off terminal maltose units. They have been positively identified only in plants. They require no ionic activators but require free -SH groups for activity.

The presence of α-amylase in normal cows' milk has been reported by Sato *(12)*, Chrzaszcz and Goralowna *(1)*, and Richardson and Hankinson *(11)*. Gould *(2)* used α-amylase in cows' milk as an index of the efficiency of pasteurization. Schloemer *(13)* found that both Ca^{++} and Cl^- were necessary for maximum activation of the enzyme in dialyzed lead sera from milk. All of these authors measured α-amylase activity by the Wohlgemuth *(17)* starch-iodine method, which is a measure of dextrinization, but Richardson and Hankinson *(11)* also demonstrated liquefaction by viscosity measurements, and noted an increase in reducing sugars when starch and milk were incubated together for 24 hr. They attributed this saccharifying power to a separate enzyme (β-amylase), since it persisted in milks heated at 65° C. for 30 min.; whereas, the loss in starch-iodine color was not observed in milks heated at 50° C. or over

Received for publication January 10, 1957.

[1] Paper No. 3710, Scientific Journal Series, Minnesota Agricultural Experiment Station. The data for this paper are taken from a thesis presented by E. J. Guy in partial fulfillment of the requirements for the Ph.D. degree, June, 1955.

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for 30 min. Inasmuch as α-amylase also produces free sugar, it is difficult to explain the observation of Richardson and Hankinson, that the saccharifying power was not diminished at all by heating the milk sufficiently to inactivate the dextrinifying power completely (55° C. for 30 min.). Schloemer *(14)* was unable to detect the presence of β-amylase in cows' milk, finding only a zone corresponding to α-amylase when a lead serum from milk was allowed to diffuse into a starch-containing gelatin gel which was subsequently stained with iodine.

Since the physical and chemical properties of the α-amylase of milk are not well defined, a more detailed study of this enzyme was undertaken. For this purpose, the enzyme was concentrated by fractionation of the milk proteins.

MATERIALS AND METHODS

Starch substrate. Raw white potato starch was obtained from a lot prepared by Loewus *(7)*. It contained 14.7% moisture (determined by vacuum oven drying at 105° C.). From this a standard stock starch paste containing 4.25% starch (wet weight basis) was prepared, according to a modification (no buffers added) of the method of Landis and Redfern *(6)*.

Milk source. All milk was obtained from the University herd or from the Twin City Milk Producers' Association. Samples used for fractionation were processed immediately upon receipt. Milk samples for assay were sometimes stored under toluene at 3° C. for periods up to a week.

Procedure for measuring liquefaction. Alpha-amylase activity was measured primarily by the method of Hultin *(5)*, in which the activity per unit of enzyme-containing material is calculated from the rate of change of the reciprocal of the specific viscosity as follows:

$$\mu\text{A/g or } \mu\text{A/ml} = \frac{d\eta_{sp}^{-1}}{dt} \times C^2 \times 10^6 \times \frac{V}{a} \qquad (1)$$

where: μA = microactivity unit

η_{sp} = specific viscosity = $\eta_r - 1$

t = time (Hultin used seconds, but in this paper the time was taken in minutes. Thus, the units reported here are 1/60th of Hultin's)

C = concentration of starch (14.7% moisture basis) in the reaction mixture in grams per ml.

a = amount of enzyme solution (grams or ml.) in the reaction mixture

V = total grams or ml. of reaction mixture.

Viscosities were measured with Ostwald viscosimeters having outflow times varying from 34.1 to 42.1 sec. with 0.025 N acetic acid at 30.0° C. Reaction mix-

tures consisted of 0.5 to 1.5 ml. of the solution being assayed, 7.1 g. of starch paste (previously tempered to the reaction temperature), and buffer and activators sufficient to make 10 ml. Thus, the initial starch concentration of the reaction mixture was approximately 3% (wet weight basis.) Exactly 8 ml. of reaction mixture was pipetted into the viscometer and incubated therein at either 34° C. ± 0.1° C. or 48° C. ± 0.1° C. Four or five outflow times were determined in seconds at intervals during a total elapsed time of from 25 to 40 min. Specific viscosities were calculated as follows:

$$\eta_{sp} = \frac{t_s - t_o}{t_o}$$

where: t_s = outflow time for assay solution

t_o = outflow time for 3% starch paste fully liquefied by concentrated bacterial amylase

The reciprocal of specific viscosity, η_{sp}^{-1} was then plotted against incubation time; in all cases such plots were linear. The slope of the plot $\frac{d\eta_{sp}^{-1}}{dt}$ was calculated and corrected by subtraction of the slope obtained with blanks containing boiled enzyme solution or water, instead of active enzyme. The corrected slope value was used to calculate activity (μA/g or μA/ml) according to Equation (1). In general, the rate of decrease of viscosity of the blank did not amount to more than from 2 to 5% of that of the test sample.

Procedures for measuring saccharification. Sugar liberated from the starch substrate was measured by the dinitrosalicylic acid method of Sumner and Howell *(16)*, and with picric acid by a modification of the method of Perry and Doan *(10)*. Standard curves for each method were made with maltose monohydrate. With either method, reducing sugar equivalent to from 0.2 to 1.0 mgm. of maltose could be determined.

The dinitrosalicylic acid method was used only with purified serum proteins. Test solutions were made with a total volume of 10 ml. of starch, buffer, and enzyme (and activators when used). Specific details of the composition of the reaction mixture, and the incubation conditions, are given in connection with individual experiments. At intervals during incubation, 1-ml. aliquots were withdrawn and added to 5 ml. of dinitrosalicylic acid reagent and 1 ml. of 0.25 *N* NaOH. This mixture was heated for 6 min. in a boiling water bath and cooled. Ten ml. of water was added and absorbance read at 540 mμ with a Coleman Model 11 Spectrophotometer. Corrections were made for the influence of such activators as cysteine hydrochloride and $CaCl_2$ on the color development. Blanks were made by separately adding the buffer, starch, and enzyme to 5 ml. of the dinitrosalicylic acid, then heating and determining absorbance as with the samples.

The picric acid method was used to measure reducing sugar produced by dialyzed skimmilk and in some cases by protein fractions. Typical test solutions consisted of 4 ml. of dialyzed milk, 5 ml. of 4.25% starch, and 1 ml. of buffer.

After incubation, exactly 2 ml. of this solution were added to 2 ml. of saturated picric acid, causing precipitation of the milk proteins. The mixture was filtered through glass wool and 2 ml. of the filtrate added to 2 ml. of 25% Na_2CO_3 and an additional 1 ml. of saturated picric acid. This mixture was heated for 20 min. in a boiling water bath, made to 10 ml. with distilled water, and the absorbance read at 520 mμ.

TABLE 1

Starch liquefying power of milk and dialyzed milk

Lot	Liquefying power[a]	
	Not dialyzed	Dialyzed
	($\mu A/ml$)	
1. Whole milk–herd	10.4	26.7
2. Whole milk–herd	20.4	31.3
3. Whole milk–commercial	13.5	13.7
Skimmilk–commercial	14.1	15.6
4. Skimmilk–commercial	14.1	16.2
5. Whole milk–commercial	17.4	17.4
Skimmilk–commercial	19.8	23.6
6. Whole milk–commercial	15.6	18.8
Skimmilk–commercial	16.7	19.9
7. Skimmilk–commercial	15.0	16.4

[a] All determined in presence of 0.06 or 0.075 *M* $CaCl_2$, 34° C., pH 7.3–7.4 (THMAM buffer), 3.0% starch substrate.

Procedure for measuring dextrinization. The dextrinization produced by purified α-amylase was measured by a modification of the method of Smith and Roe *(15)*, in which the concentration of starch remaining at any time is determined colorimetrically by its reaction with iodine. The specific conditions used were: pH 7.40, tris (hydroxymethyl) aminomethane (THMAM) buffer of ionic strength 0.035, 0.075 *M* $CaCl_2$, 34° C., and 0.6% starch concentration. Dextrinizing power was expressed in terms of mgm. of starch hydrolyzed in 30 min., calculated as follows:

$$\text{Starch hydrolyzed in 30 min. (mgm.)} = \left(\frac{A_{\text{control}} - A_{30\text{ min.}}}{A_{\text{control}}} \right) \times 60$$

where: A_{control} = absorbance at 620 mμ for the control containing 60 mgm. starch per tube

$A_{30\text{ min.}}$ = absorbance at 620 mμ for sample incubated for 30 min.

Procedure for measuring protein content. The protein content of various fractions was routinely followed by a turbidimetric method using trichloroacetic acid as a protein precipitant. Three ml. of 50% trichloroacetic acid were added to 5-ml. aliquots of diluted protein solution in matched colorimetric tubes. At the end of 5 to 10 min., absorbance readings were taken on a Coleman 11 Spectrophotometer at 650 mμ. A standard curve was constructed with lactoglobulin relating mgm. of protein N (determined by a micro-Kjeldahl procedure) to absorbance. From 0.03 to 0.16 mgm. of protein N per tube could be determined using this method.

TABLE 2

Liquefying and saccharifying power of dialyzed milk under various conditions

Lot	Heat treatment	Incubation conditions Temp.	pH [a]	Salts added	Conc. EDTA [b]	Liquefying power	Saccharifying power [c]
		(° C.)			*(molarity)*	*(μA/ml)*	*(mgm. maltose hydrate/ hr/liter)*
1. Whole Herd	None	34	6.2	0.06M NaCl	0.0002	19.0	—
	None	34	6.2	0.06M $CaCl_2$	0.0002	22.4	—
	None	34	7.3	0.06M $CaCl_2$	0.0002	26.7	—
2. Whole Commercial	None	48	6.4	0.06M NaCl	0.0002	34.0	1,700
	None	48	6.4	0.06M NaCl	0.0023	9.0	—
	None	48	6.4	0.06M NaCl	0.0042	3.0	—
	None	48	6.4	None	0.0002	1.6	—
3. Whole Herd	None	48	6.4	0.06M NaCl	0.0002	39.0	2,500
	None	48	6.4	0.06M NaCl	0.0042	4.1	270
	57.5° C. 30 min.	48	6.4	0.06M NaCl	0.0002	2.2	120
4. Skim Herd	None	37.5	6.4	0.03M NaCl	None	—	1,380
	None	37.5	6.4	None	0.0031	—	193
	53.5° C. 30 min.	37.5	6.4	None	0.0031	—	193
	68 ° C. 30 min.	37.5	6.4	None	0.0031	—	Negligible
	None	37.5	5.1	None	0.0031	—	132

[a] pH controlled by following buffers: pH 6.2 and pH 6.4—phosphate, pH 7.3—THMAM, pH 5.1—acetate. The concentration of buffer in reaction mixture was $\mu = 0.1$.

[b] Concentration of disodium ethylene diamine tetraacetate in incubation mixture.

[c] Measured by picric acid method.

EXPERIMENTAL PROCEDURE

Liquefying and saccharifying activities of dialyzed milks. Although the liquefying power of unmodified milk can be determined readily, direct measurement of its saccharifying power would necessitate a large correction for the lactose. Furthermore, bacterial growth might occur at the incubation times and temperatures used. Consequently, milks were dialyzed in the presence of toluene at 3° C. *vs.* 5×10^{-4} *M* disodium ethylenediamine tetraacetic acid (EDTA) until virtually free of lactose, and their liquefying and saccharifying powers measured. Results are shown (Tables 1 and 2).

Dialysis markedly increased the liquefying activity of two samples, but did not greatly affect that of eight others; no explanation is evident for this difference. Heat treatment at 57.5° C. for 30 min., or further addition of EDTA, suppressed liquefaction and saccharification in a like manner. Heat treatment very likely denatures the enzyme. Since all of the Ca^{++} probably was not removed by the dialysis, and since calcium and chloride are both necessary for the activity of the α-amylase *(13)*, the effect of the additional EDTA probably was due to sequestration of this ion. For some, but not all dialyzed milks, a small but measurable saccharification was observed in the presence of EDTA and absence of any activators (Sample 4, Table 2). This activity, which appears to represent a β-amylase, was carefully measured by using as controls both milk heated to 68° C. for 30 min. and a mixture of separately incubated milk and starch plus buffer. Samples were incubated for from 12 to 24 hr. under toluene, to measure this low activity.

TABLE 3

Distribution of alpha-amylase activity among whole milk, skimmilk, acid whey, lactoglobulin, and lactalbumin

Lot	Liquefying power [a] (*μA per ml. whole milk*)				
	Whole	Skim	Acid whey	Lactoglobulin [b]	Lactalbumin [c]
1. Mixed milk, 4 cows	.....	9.9	.....	4.5	3.8
2. Mixed milk, commercial	19.0	19.7	.... .	.... .	.....
3. Mixed milk, commercial	13.5	14.1	9.1	6.1	Negligible
4. Mixed milk, commercial	17.4	19.8	16.2	6.2	Negligible
5. Mixed milk, commercial	15.6	16.7	9.5	10.4	Analysis unsatisfactory
6. Mixed milk, commercial	.....	15.0	8.9	9.4	1.0

[a] All determined in presence of 0.06 or 0.075 *M* $CaCl_2$, 34° C., pH 7.3–7.4, (THMAM buffer), 3.0% starch substrate.

[b] Precipitate obtained from whey protein at pH 6.2, 43% saturation with $(NH_4)_2SO_4$.

[c] Filtrate remaining after precipitation of lactoglobulin.

Distribution of alpha-amylase in milk protein fractions. Fractionation experiments in an attempt to concentrate the α-amylase were performed with small samples of mixed milk (Table 3). The α-amylase was concentrated in the skimmilk upon separation, and in the whey upon precipitation of the casein with acid, although some of it followed the casein. Furthermore, it came down to a large extent with the lactoglobulin fraction upon precipitating the latter at pH 6.2

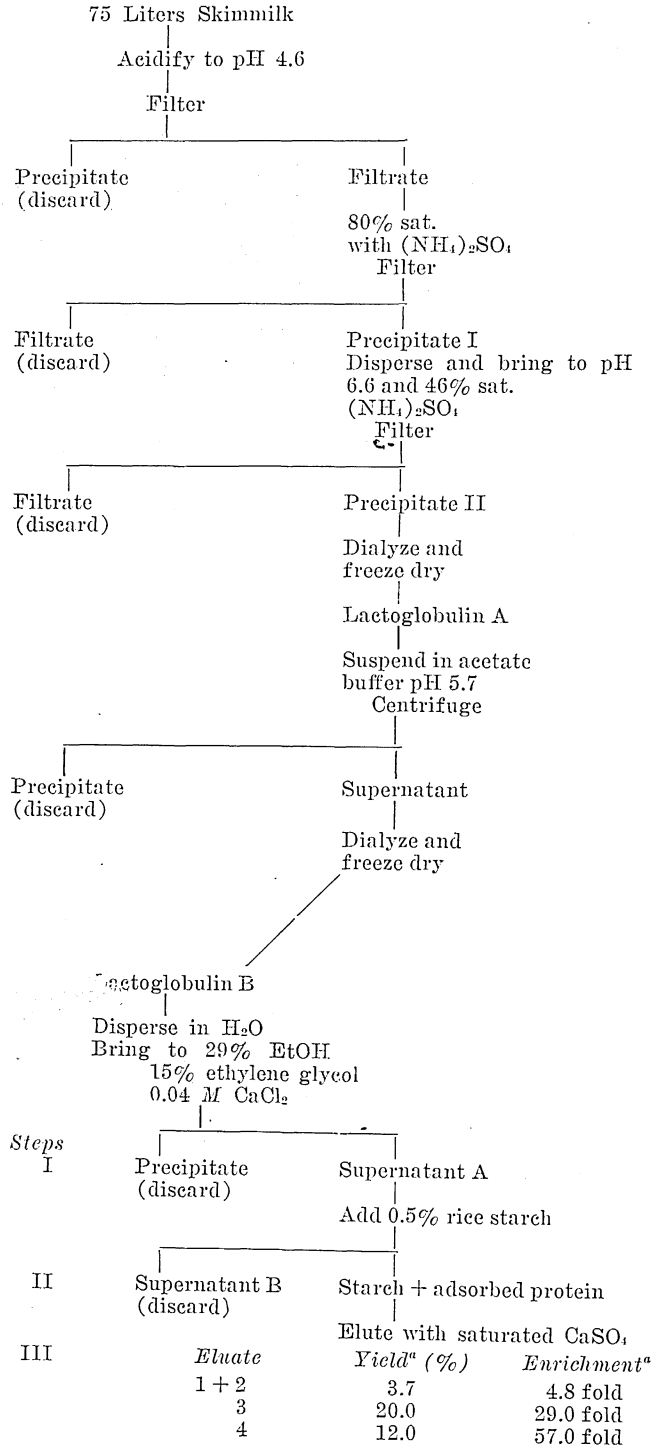
with 43% saturated ammonium sulfate, although in one case (Lot 4) considerable enzyme was lost in this fractionation. Thus, the lactoglobulin fraction was used as the starting material in attempts to concentrate and purify the enzyme.

Concentration of α-amylase from lactoglobulin. Concentration of α-amylase was based upon the work of Holmbergh *(4)*, in which the enzyme was dissolved in 30 or 40% ethanol, adsorbed upon rice starch at 0° C., and eluted from the starch by shaking with water. When this procedure was applied to lactoglobulin, only from 14 to 38% of the α-amylase could be dissolved in the ethanol solution. However, inclusion of 15% ethylene glycol (v/v) solubilized up to 86% of the α-amylase. Moreover, when rice starch was added to this mixture, up to 94% of the solubilized α-amylase was adsorbed on it, and when eluted from the starch with water, the α-amylase was concentrated up to 57-fold (12% yield based on activity of the lactoglobulin). With one sample, an enrichment of 240-fold over the activity of the lactoglobulin was obtained by two successive adsorptions and elutions.

Samples of the lactoglobulin fraction were isolated in large amounts and assayed for α-amylase. On the basis of preliminary kinetic studies of these samples, α-amylase activity was followed during fractionation by determining liquefaction at 48° C. on 3.20% potato starch paste at pH 6.2 with acetate buffer ($\mu = 0.01$) in the presence of 0.06 *M* $CaCl_2$. A lot of lactoglobulin containing a relatively high concentration of α-amylase and some apparent β-amylase activity was chosen for further fractionation.

The steps employed in the purification of α-amylase are summarized in Figure 1 and in the following paragraphs. Seventy-five liters of skimmilk from the University herd was acidified to pH 4.6 by slowly adding 4 liters of 1 *N* HCl with stirring. The next morning the casein was filtered off through washed cotton sacks and filter paper. To 58 liters of the whey, 71.1 lb. of $(NH_4)_2SO_4$ (80% saturation) were added. After being held overnight, the mixture was filtered and the precipitate, I, dispersed in water to give 16 liters of total volume. Ten liters of saturated $(NH_4)_2SO_4$ was added (46% saturation) and the pH adjusted to 6.6 with 1 *N* NaOH. The mixture was filtered and the precipitate, II, was held 4 wk. at 3° C. under toluene. It was then exhaustively dialyzed against both tap and distilled water until free of $SO_4^=$. The contents were frozen, pulverized, and freeze-dried in an apparatus described by Nickerson, Coulter, and Jenness *(9)*. Eighty-seven grams of the material thus obtained (designated Lactoglobulin A) was stored at 3° C. for a month. The preparation was suspended in acetate buffer ($\mu = 0.1$), pH 5.7, and allowed to stand for 2 hr., following which it was centrifuged to remove a small amount of a lipoprotein fraction. The supernatant, containing the active enzymes, was filtered through qualitative filter paper, exhaustively dialyzed under toluene against distilled water at 3° C., and again freeze-dried. Seventy grams of this purified lactoglobulin (designated Lactoglobulin B) were obtained. It had an α-amylase activity of 25.0 μA/mgm *N* and 1.2 mgm. maltose hydrate/hr/mgm *N* at 48° C. and pH 6.2. It was fractionated further by the following steps:

FIG. 1. Purification of Alpha-Amylase.



[a] Yield and enrichment calculated on basis of Lactoglobulin B.

Step I. About 16.0 g. of Lactoglobulin B were suspended in 610 ml. of 0.08 *M* $CaCl_2$ solution at 3° C., and 180 ml. of ethylene glycol at 3° C. was slowly added. The next morning, 420 ml. of 83% ethanol (v/v) precooled to 3° C. was added dropwise from a separatory funnel over a 15-20 min. period while stirring the mixture. After 4 hr., the mixture was centrifuged at high speed for 15 min. in a Servall centrifuge and the precipitate washed twice with 100 ml. of the solution containing 29% ethanol and 15% ethylene glycol. The supernatant (designated A) contained 86% of the total α-amylase (enrichment 1.1-fold) and 96% of the β-amylase (enrichment 1.2-fold).

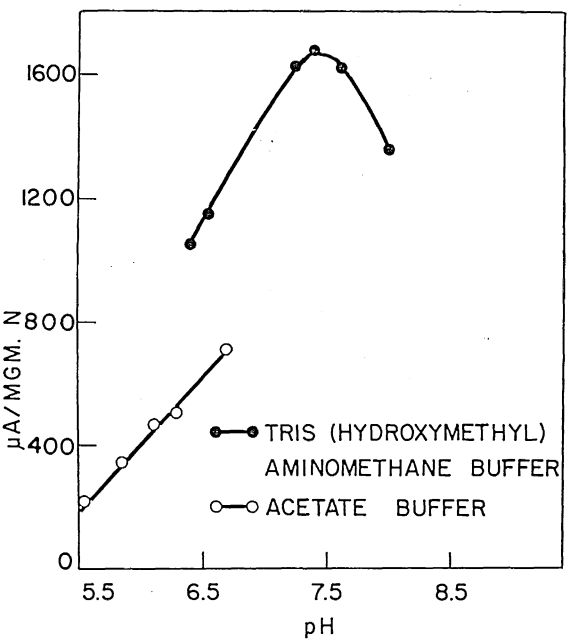


FIG. 2. Effect of pH on liquefying activity of purified alpha-amylase.

Starch concentration	= 3.20%
$CaCl_2$	= 0.075 *M*
Temperature	= 34° C.
Reaction time	= up to 30 min.

Step II. Approximately 6.0 g. of pulverized rice starch (Morningstar Nicol, Inc.) was added to 1,200 ml. of the Supernatant A; two days later the starch was centrifuged at 2,500 r.p.m. for from 20 to 30 min. The supernatant (designated B) contained 93% of the total β-amylase and 17% of the total α-amylase activity.

Step III. The starch, carrying 69% of the α-amylase, was eluted with four portions of saturated $CaSO_4$ solution. After each elution, the starch was centrifuged and the supernatant, containing the α-amylase, was dialyzed. The first elution was made by stirring the starch with 30 ml. of solution for about a minute. The second elution was made by stirring for one-half hour with 50 ml. of solu-

tion. The third and fourth elutions were made by stirring for periods of up to an hour with 75 ml. of solution. The yield of enzymatic activity is shown (Figure 1).

Properties of Concentrated Enzyme. A lot of purified α-amylase, prepared by the scheme outlined in Figure 1, was chosen for further study. It was enriched about 30-fold, compared to the lactoglobulin from which it was isolated, having a liquefying activity at 34.3° C. and pH 7.4 of 1,750 μA/mgm *N*, saccharifying activity of 149 mgm. maltose hydrate/hr/mgm *N*, and dextrinifying activity of 115 mgm. starch 30/min/mgm *N* (11.5 Smith and Roe units per mgm *N*).

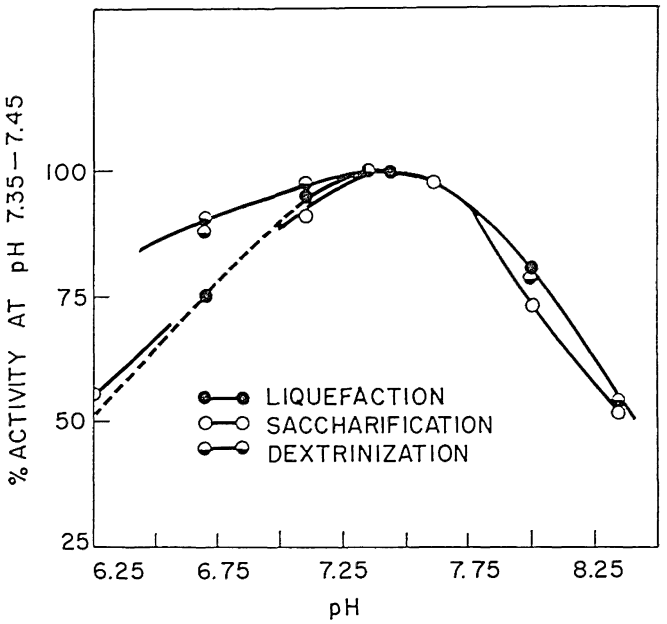


FIG. 3. Effect of pH on saccharifying, dextrinizing, and liquefying activities of purified α-amylase.

Starch concentrations = saccharification 1.12%, dextrinization 0.6%, liquefaction 3.20%
$CaCl_2$ = 0.075 *M*
Temperature = 34° C.
Reaction times = Saccharification to 65 min., dextrinization to 80 min., liquefaction to 30 min.
Buffer = Tris(hydroxymethyl)aminomethane (THMAM)

A preliminary check of the effect of temperature on the liquefying action of this lot was made at pH 6.2, acetate buffer, $\mu = 0.1$, 3.20% starch, 0.06 *M* $CaCl_2$, and 30 min. reaction time. This series indicated an optimum temperature of about 44° C. Hence, in further work, a temperature of 34° C. was adopted, in order to avoid complications due to inactivation of the enzyme.

Effect of pH. The effect of pH on the liquefying activity was determined at 34° C. over the range from 5.5 to 8.0, with acetate and THMAM buffers. The reaction mixtures had a buffer ionic strength of 0.1 and $CaCl_2$ concentration of 0.075 *M*. The data (shown in Figure 2) indicate optimum activity at pH 7.4.

The reason for the greater activity in THMAM buffer at pH 6-6.5, as compared to acetate at the same pH, is not known, although such effects are often noted in enzyme chemistry.

The effect of pH on the saccharifying and dextrinifying activities was also determined with THMAM buffers. The results (plotted in Figure 3) indicate that the optima for all three measures of activity coincide at pH 7.4.

Effect of temperature and energy of activation. The liquefying activity of the α-amylase preparation was determined at temperatures between 25 and 49° C. at pH 7.4. In all cases, the plots of $1/\eta_{sp}$ *vs.* time were linear up to 30 min. reaction time, which is indicative of a zero-order reaction. A plot of log of the reaction rate (actually, log μA, which is proportional to the log rate) *versus* the reciprocal of the absolute temperature, exhibits (Figure 4) a linear relationship

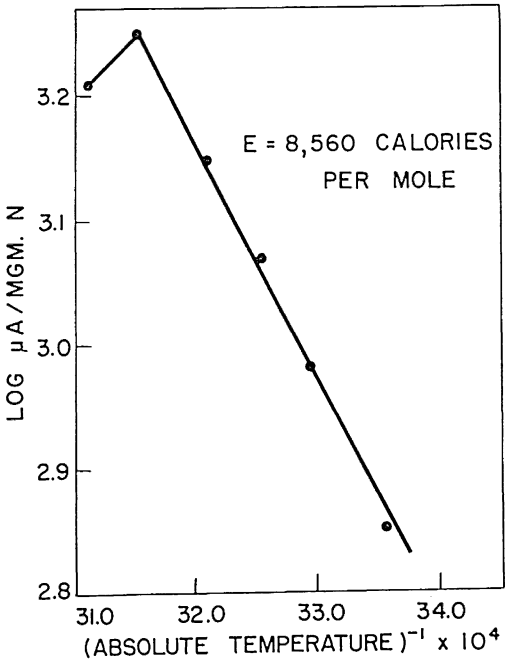


FIG. 4. Effect of incubation temperature on the rate of liquefaction of starch by purified α-amylase.

Starch concentration	= 3.2%
$CaCl_2$	= 0.06 *M*
pH	= 7.3—7.4 (THMAM buffer)
Reaction time	= up to 30 min.

up to 44° C., beyond which denaturation causes a reduction in activity. The energy of activation, calculated from this plot by the Arhennius equation, log rate = $-E/2.303\,RT + C$, is 8,560 calories per mole.

Effect of heat treatment. A lot of the purified enzyme and two lots of lactoglobulin were heated in solution in acetate buffer at pH 6.4, $\mu = 0.1$ for 30 min. at various temperatures. Likewise, a sample of skimmilk was given similar heat treatments. Liquefying activities as percentages of the activity of the respective

unheated controls are plotted against temperature (Figure 5). All four samples exhibited a sharp reduction in activity in the range 45-52° C., with half of the activity being destroyed by heating at about 50° C. for 30 min.

Effect of substrate concentration. The effect of starch concentrations ranging from 0.06 to 0.6% on the rate of saccharification was determined. A plot of these data by the method of Hofstee *(3), V. vs. V/S,* yielded a straight line (shown in Figure 6). Linearity of such a plot indicates that only one enzyme is present. In the Hofstee plot, the slope is equal to $-K_m$, the Michaelis constant, and the intercept on the ordinate is the maximum velocity. For this α-amylase preparation, K_m is 2.05×10^{-3} g. starch/ml on a 14.7% moisture basis, or 1.75×10^{-3} g. dry starch/ml. The maximum velocity is 140 mgm. maltose hydrate/hr/mgm N.

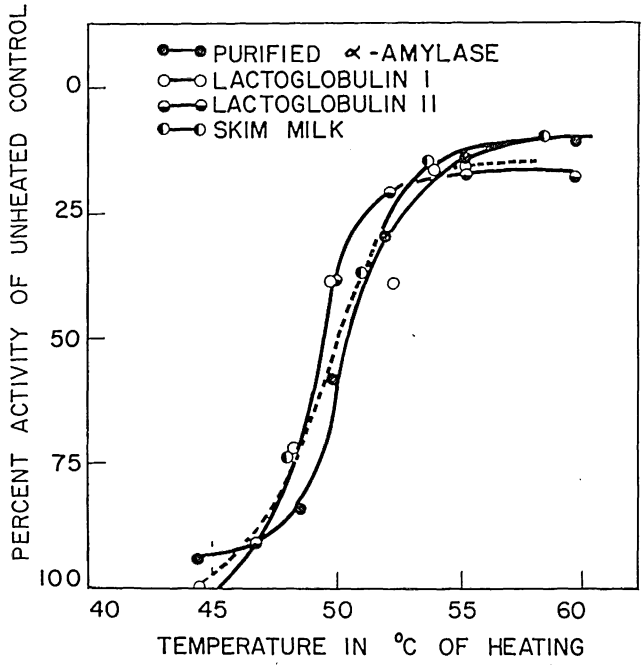


FIG. 5. Effect of 30-min. heat treatments upon the starch liquefying activity of purified α-amylase, lactoglobulin, and skimmilk.

Starch concentration	= 3.2%
$CaCl_2$	= 0.06 M
pH	= 7.10 (THMAM)
Temperature	= 34° C.
Reaction time	= up to 30 min.

The effect of substrate level on the rate of liquefaction by this amylase preparation was also studied. The activity appeared to increase at starch concentrations below 2%, but the viscosity method is not very sensitive at lower than 1% starch. Thus, no measurement of K_m could be made by this method.

Effect of activators on the liquefying and saccharifying activity. The effect of $CaCl_2$, calcium acetate, iodine, NaCl, $MgCl_2$, and cysteine HCl upon the liquefying and/or saccharifying activity of α-amylase was determined. The

results (shown in Table 4) indicated that optimal activation is attained for liquefaction by 0.075 *M* $CaCl_2$; NaCl and $MgCl_2$ of comparable ionic strength activate the enzyme to only 10% of the level attained with $CaCl_2$. Calcium acetate is without activation effect. Data also indicate that NaCl, or calcium acetate, activates no saccharification, whereas $CaCl_2$ does. However, the apparent total absence of activity may be a reflection of the dinitrosalicylic acid method used, in which small levels of sugar (0.18 mgm. maltose hydrate) are not detected. The addition of iodine to the starch substrate decreased the effects of both the liquefying and saccharifying activities by approximately the same amount, respectively, 33 and 31%. Cysteine did not elicit additional saccharifying activity.

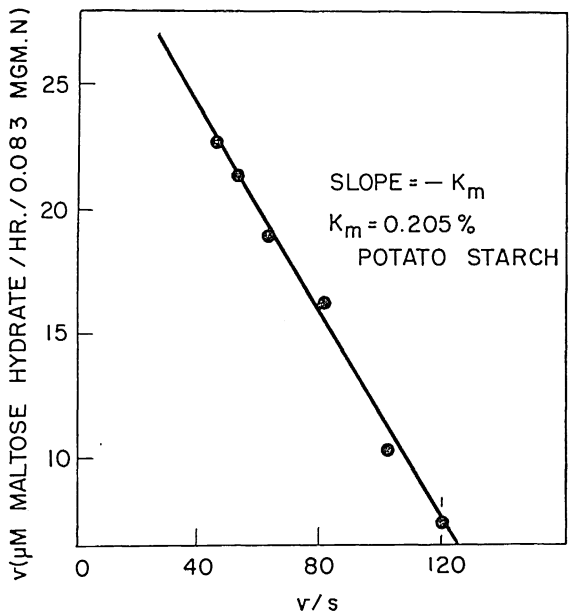


FIG. 6. Plot of velocity *vs.* velocity/substrate concentration for saccharifying activity of purified α-amylase.

Starch concentrations	= 0.06 — 0.60%
$CaCl_2$	= 0.5 *M*
pH	= 7.4 (THMAM)
Temperature	= 34° C.
Reaction time	= up to 120 min.

All velocities extrapolated to zero-time.

DISCUSSION

This work confirms that of Chrzaszcz and Goralowna *(1)*, Sato *(12)*, Schloemer *(13)*, and others in showing that cows' milk contains an α-amylase. It supports the findings of Richardson and Hankinson *(11)* by indicating that at least some lots of cows' milk contain weak β-amylase activity.

The present findings also corroborate those of Schloemer *(13)* in showing that both Ca^{++} and Cl^- ions are necessary for the activation of this α-amylase, and that it largely follows the whey protein fraction of milk. Furthermore, the

reports of previous workers *(11, 13)*, that this is a very heat-labile enzyme, have been confirmed. This heat lability was demonstrated both in milk and in concentrated preparations.

The purified α-amylase prepared was believed to be free from β-amylase, because *(1)* no saccharification was detected in the absence of both calcium and chloride, *(2)* a plot of velocity *vs.* velocity/concentration of substrate, according to the method of Hofstee, yielded a straight line indicative of one component, *(3)* in the fractionation of lactoglobulin negligible β-amylase was adsorbed upon rice starch, *(4)* negligible additional saccharification was observed when cysteine hydrochloride, a sulfhydryl activator, was included in addition to the $CaCl_2$, and *(5)* the decrease in liquefaction caused by the iodine was paralleled by the decrease of saccharification.

TABLE 4

Effect of different activators and inhibitors on the liquefying and saccharifying activity of purified alpha-amylase

Adjunct[a]	pH[b]	Ionic strngth[c]	Activity[d]	
			(μA/mgm N)	*(mgm. maltose hydrate/hr/ mgm N)*
1. None	7.65	0.035	None	None
2. NaCl—0.10 *M*	7.65	0.135	171	None
3. $MgCl_2$—0.075 *M*	7.4	0.260	196	.....
4. $CaCl_2$—0.060 *M*	7.4	0.215	1690	.....
5. $CaCl_2$—0.075 *M*	7.4	0.260	1750	149
6. $CaCl_2$—0.090 *M*	7.4	0.305	1740	.....
7. Ca acetate—0.075 *M*	7.4	0.260	None	None
8. Cysteine HCl—0.0008 *M* +$CaCl_2$—0.075 *M*	7.4	0.261	.....	152
9. Iodine—KI—.00024 *M* I_2+$CaCl_2$—0.075 *M*	7.4	0.260	1170	103

[a] Concentration in reaction mixture.

[b] The effect of Ca and Mg on the pH of the buffer was not compensated.

[c] Including THMAM buffer of ionic strength $\mu = 0.035$. In Experiments 1 and 7 the THMAM buffer was used as the acetate, in all other experiments as the chloride.

[d] All incubations at 34° C. Dinitrosalicylic acid method used for saccharification; 3.20% starch and 30 min. incubation used for liquefaction, 1.50% starch and 150 min. incubation used for saccharification.

Alpha-amylase from cows' milk differs from those from other sources by its dependence upon both the chloride and calcium ions for maximal activity. Those from plant sources require calcium; whereas, in general, those of animal or microbial source require chloride ions. The pH optimum of 7.4 found for the milk enzyme resembles that of amylase from human blood (6.8–7.6).

Large amounts of milk would have to be worked up if further purification of the α-amylase were to be attempted. Thus, one liter of milk containing 18,000 μA units yields, assuming a 50% loss, about 1.25 g. of lactoglobulin, with a specific activity of about 45 μA/mgm *N*. This lactoglobulin yields, in turn, with about 60% loss (see Figure 1), a concentrate enriched about 40 times with a specific activity of about 1800 μA/mgm *N*. Even this concentrate has a specific activity of only 1/200th to 1/500th that of crystalline α-amylases from other sources.

Although glycerol is known to be a good solubilizing agent for enzymes, the authors are not aware of the previous use of ethylene glycol for this purpose. It proved very effective in controlling the solubility of α-amylase in ethanol solution. Moreover, by this addition, the concentration of the ethanol could be kept high enough to cause adsorption of the enzyme upon added starch.

ACKNOWLEDGMENT

The financial support of this work by the American Dry Milk Institute, Inc., is gratefully acknowledged.

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DETERMINATION OF UNOXIDIZED TOCOPHEROLS IN MILK FAT[1,2]

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SUMMARY

This procedure was evolved in order to follow tocopherol degradation in oxidizing milk fat. It combines saponification, in the presence of pyrogallol, to destroy tocoquinones and essentially all fat peroxides, chromatography on $SnCl_2$ + HCl-treated Floridin (Kjolhede) to remove carotenoids, possible residual peroxides, steroids, etc., and color development in benzene-ethanol with ferric chloride and bipyridyl in acetic acid solution (Devlin and Mattill). Pyrogallol was effective in preventing tocopherol destruction during saponification and was superior to saponification under nitrogen. Complete saponification was effected by using 17 ml. of 3.5 *N* KOH per 10 g. fat. If saponification was eliminated, the treated Floridin column reduced tocopheryl-*p*-quinones but not tocopheryl-*o*-quinones. The former were similarly reduced by the Quaife and Biehler hydrogenation procedure. These reduced compounds are measured as tocopherols.

A method was needed for the determination of unoxidized tocopherols present in milk fat, in order that their destruction during oxidation of milk fat samples in storage could be followed. It was likewise considered that the amount of destruction of tocopherols might be employed as a criterion of degree of oxidation of the fat.

A literature survey indicated that many methods had been employed for tocopherol determination in fatty materials. The most common was that of Emmerie and Engel *(5)*, together with its many modifications. The procedure that will reported combines certain aspects of those of Devlin and Mattill *(3)*, Moore and Tosic *(8)*, and Kjolhede's modification *(7)* of Emmerie and Engel's method *(5)*.

EXPERIMENTAL PROCEDURE

In the search for simple methods, those of Quaife and Biehler *(10)*, Parker and McFarlane *(9)*, and Kjolhede (with whole fats) *(7)* were tried. The first was unsatisfactory because of the reduction of tocoquinones present in the oxidized fat; the second caused troublesome emulsions with some fats, and the third yielded high values with oxidized fats, presumably because of reduction of tocoquinones on the column.

The reason for selecting the combination indicated above resulted from the fact that Devlin and Mattill's *(3)* use of benzene-acetic acid in place of ethanol caused slower color development, improved lipid solubility, and eliminated

Received for publication May 14, 1957.

[1] Journal Paper No. J-3208 of the Iowa Agricultural Experiment Station, Ames. Project No. 982.

[2] The work reported herein was supported in part by the Quartermaster Food and Container Institute for the Armed Forces; the paper has been cleared for publication.

[3] Part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology–Dairy Chemistry, December, 1950.

[4] Current address: Kraft Foods Co., Research Laboratories, Glenview, Illinois.

troublesome light-catalyzed oxidation of ethanol by ferric iron. Interfering tocoquinones and peroxides were removed by saponification of the sample in the presence of pyrogallol *(8)*. Interfering carotenoids, vitamin A, steroids, and possibly other materials were removed from benzene solutions of nonsaponifiables by use of specially treated Floridin *(7)*.

Tocopherol determinaton. Saponification and extraction of sample. Mix 10 g. of fluid fat and 5 ml. pyrogallol solution (5 g. to 100 ml. reagent absolute methanol) in ST. 150-ml. actinic extraction flask, add 20 ml. 3.5 *N* absolute methanolic KOH, reflux on an 80° C. water-bath for 10 min. after the solvent begins to drip from the condenser tip. Remove heat, rinse condenser into saponification mixture with 5 ml. absolute methanol, remove flask, add 40 ml. distilled H_2O, mix, add 15 ml. absolute methanol, stopper, and cool in a room-temperature water-bath. Transfer to a 250 ml., g. s., amber separatory funnel, rinse saponification flask with small portions (aggregate 60 ml.) distilled water, and small portions (aggregate 80 ml.) of peroxide-free ether.[5] Shake vigorously ten times, remove ether layer, and extract the aqueous layer twice more with 50 ml. peroxide-free ether. Combine extracts and wash successively, with 50 ml. 1% aqueous KOH, 50 ml. 2% Calgon, and two 50-ml. portions of distilled water. Dry over Na_2SO_4. Remove ether from dried solution under vacuum, introduce 2 ml. benzene[6] through the stopcock of the evaporation apparatus *(14)*, release vacuum, and make residue to volume in a 25-ml. volumetric flask.

Tocopherol analysis. Prepare Floridin columns,[7] chromatograph benzene solutions of nonsaponifiables (equivalent to 4 g. fat), and evaporate combined percolate and washings by the Kjolhede method *(7)*, except that in eluting the columns use five, 5-ml. portions of benzene.

To develop the ferrous-bipyridyl color, transfer the residue from the last evaporation of the percolate and washings, quantitatively to a 25-ml., actinic red, g. s., graduated cylinder with benzene, make to the 10-ml. mark with benzene, add 2 ml. of Devlin and Mattill reagent *(3)*, make to 25 ml. with absolute ethanol,[8] invert three times, pour into a cuvette, and read the percentage transmittance (Coleman Model 11 Spectrophotometer) at 520 mμ, 5 min. after the last of the reagent is delivered from the pipette, against a blank (prepared

[5] Peroxides were determined by a modified Chapman and McFarlane method *(1)*: Dissolve 0.2 g. NH_4SCN in 2 ml. distilled H_2O and make to 50-ml. volume with purified acetone. Invert flask into a folded Whatman No. 40 filter containing 0.1 g. $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$. Catch filtrate in a second 50-ml. amber volumetric flask. Place 5 ml. reagent and 5 ml. purified acetone in a color-matching tube as the blank; use 5 ml. reagent and 5 ml. ether in a second tube as the peroxide test.

[6] Reagent, thiophene-free benzene, was refluxed over 20 g. KOH and 5 g. aluminum per liter and distilled; the first 50 to 100 ml. of a 4-liter batch were discarded.

[7] The Floridin XXS used was furnished by The Floridin Co., Warren, Pa. The tubes employed were 12-cm. pieces of 10-mm. tubing, joined at one end to 5-cm. pieces of 5-mm. tubing and at the other to 10-cm. pieces of 16-mm. tubing.

[8] Refluxed over 20 g. KOH pellets and 2 g. $KMnO_4$, per liter for 1 hr.; discard the first and last 300 ml. of 4-liter batch.

without sample) set at 100% transmittance. The tocopherol quantity can be calculated by use of a standard regression equation.[9]

Milk fat was prepared from unsalted butter. The butter was churned from 40% cream (obtained from the Iowa State College Market Milk Department) in quart Mason jars with glass lids and rubber gaskets, in a shaker with reciprocating action in the direction of the long axis of the jar. After sharp-freezing for 24 hr., the butter was melted (40-45° C.), centrifuged to separate the fat from the aqueous phase, the fat filtered through Whatman No. 12 folded filters, in heated funnels (fat temperature *ca.* 45° C.), and collected and stored in 4-oz. aluminum, screw-capped, glass sample bottles. Fats were stored at approximately −25° C. until used. The samples which showed peroxide values (Table 1) had been in storage for from 12 to 15 mo.

TABLE 1

Comparison of the saponification procedure employed in the vitamin A method with that employing 3.5 N methanolic KOH

Sample aliquot	Saponification procedure	Pyrogallol added	Tocopherol value (γ/g fat) Kjolhede	Peroxide value (m.e/kg) Kjolhede	Peroxide value (m.e/kg) Untreated[a]
1	Vitamin A[b]	+	46.4–46.0	0.00	0.012
2	Vitamin A[b]	+	45.8–46.7	0.00	0.007
3	Vitamin A[b]	0	30.6–31.2	0.00	0.012
4	Vitamin A[b]	0	31.0–32.2	0.00	0.009
5	Not saponified	0	55.4–56.6	0.00	3.19
6	3.5 *N* KOH	+	46.7–45.8	0.00	0.010
7	3.5 *N* KOH	+	44.9–46.0	0.00	0.015
8	3.5 *N* KOH	0	32.4–32.8	.....	.....
9	3.5 *N* KOH	0	29.6–30.2	.....	.....
10	Not saponified	0	57.3–55.8	0.00	3.02

[a] Nonsaponifiables or whole fat not passed through absorbent column.
[b] Methanolic KOH, 4 *N*.

RESULTS AND DISCUSSION

Preliminary use of the Emmerie *(4)* saponification technique indicated that it yielded various degrees of saponification (indicated by volumes of nonsaponifiable residue) and erratic results. Chipault *et al. (2)* indicate that 17 ml. of 3.5 *N* methanolic KOH were found to leave but a few drops of nonsaponifiables from 10 mg. of milk fat. A comparison of 3.5 *N* and a 4.0 *N* (U. S. Technical Committee on Vitamin A Researches) methanolic KOH indicates that results with both alkalies are essentially the same (Table 1). These data likewise confirm the protective action of pyrogallol, and show that the saponification procedure destroys most of the interfering peroxides in the fat.

Difficulties were encountered in obtaining adequate recoveries of D-α-tocopherol, when added to milk fat as a solution in benzene. When added directly to milk fat as the solvent, satisfactory recoveries (from 90 to 94%) were obtained.

[9] Standards were prepared from pure D-α-tocopherol (Distillation Products, Inc.). Benzene solutions containing 2 to 400 γ were treated for color development as described in "Tocopherol Analysis." Regression equations were calculated from the data.

TABLE 2

Comparison of the effects of nitrogen and pyrogallol on tocopherol contents of milk fat

Sample aliquot	Saponification treatment		Tocopherol (γ/g fat)	
	Pyrogallol	Nitrogen	Chromatographed [a]	Not chromatographed [b]
1	+	−	39.4	41.1
2	+	+	42.5	40.7
3	−	+	32.2	.....
4	−	−	25.7	22.9
5	+	−	44.0	41.5
6	+	+	44.0	39.5
7	−	+	24.4	22.4
8	−	−	26.6	25.9

[a] Nonsaponifiables passed through Kjolhede-Floridin column.
[b] Nonsaponifiables run without passing through column. Carotene corrections made by method of Quaife *et al. (11)*. These samples were fresh fats in which no oxidation had occurred.

TABLE 3

Degree of interference of p-quinone in the tocopherol determination

Treatment	γ *p*-quinone added per g. of fat	γ tocopherol per g. of fat	Difference (sample minus control)	% conversion of *p*-quinone added	% error (basis, 65.4 mcg. per g.)
Kjolhede	71.2	103.2	37.8	53.0	57.9
Kjolhede	35.6	93.6	28.2	79.3	43.2
Kjolhede	17.8	74.0	8.6	48.4	13.2
Kjolhede[b]	0.0	65.4	.....	.....	.....

[a] Whole fat passed through Kjolhede-Floridin columns.
[b] Control.

TABLE 4

Effect of saponification on the degree of interference of added p-quinone in the tocopherol determination

Sample aliquot	5 ml. of 5% pyrogallol added	γ *p*-quinone added per g.	Tocopherol (γ/g fat)	
			Kjolhede	Average
1 (Saponified)	+	40	54.0	53.2
2 (Saponified)	+	40	51.5	
3 (Saponified)	+	0	49.2	47.9
4 (Saponified)	+	0	46.6	
5 (Saponified)	−	40	34.0	34.0
6 (Saponified)	−	40	.....	
7 (Saponified)	−	0	33.0	34.5
8 (Saponified)	−	0	36.0	
9 (Whole fat)	−	0	51.3	
10 (Whole fat)	−	66	68.2	

Initially, it was the intent to minimize oxidative effects by saponifying samples in a nitrogen atmosphere. Erratic results were obtained. The use of pyrogallol *(8)* eliminated this variability (Table 2). These data, obtained with fresh fats, indicate that a nitrogen atmosphere is not necessary when pyrogallol is employed. A comparison of the values obtained for saponified and chromatographed fat aliquots, and those obtained for saponified aliquots which were not chromatographed but were corrected for carotene interference *(11)*, indicated that chromatography of the nonsaponifiables from fresh fats corrects only for carotene interference.

TABLE 5

Assay[a] of hydrogenated solutions of p-quinones

γ of *p*-quinone	Standing time *(min.)* after hydrogenation	γ found as tocopherol	% recovery of added *p*-quinone
333	5	157.5	47.3
333	10	134.4	40.4
333	15	161.3	48.4
333	60	68.9	20.7
333	120	44.5	13.4

[a] Quaife and Biehler method *(10)*.

Kjolhede-treated Floridin removes peroxides[10] from fat or nonsaponifiable fractions (Table 1). However, when whole oxidized fats are passed through these columns, tocopherol values are obtained that are higher (Aliquots 5 and 10) than when these fats are saponified with pyrogallol and then chromatographed. It was considered that the elevation of the values might result from reduction of *p*- or *o*-quinones[11] resulting from tocopherol oxidation. Consequently, natural D-α-tocopherol was oxidized with ceric sulfate *(12)* to produce the *p*-quinone, and by nitric acid *(13)* to produce the *o*-quinone. The *p*-quinone itself did not react with the Devlin and Mattill reagent, but when added to milk fat in quantities which might be found in oxidizing milk fat, assays of whole fat passed through Floridin columns increased by amounts equivalent to from 13.2 to 57.9% of the tocopherols naturally present (Table 3). The *o*-quinone, however, caused no appreciable difference in whole fat assays. Saponification of samples, to which the *p*-quinone was added, eliminated appreciable interference by the *p*-quinones (Table 4).

The data of Table 5 confirm the statement that the *p*-quinones are reduced to a considerable degree in the Quaife and Biehler *(10)* hydrogenation and are measured as tocopherols. They likewise indicate that the hydroquinones presumably formed are slowly oxidized.

[10] Peroxides were determined by the Hills and Thiel method *(6)*, except that the reaction mixture was heated at 60° C. for 3 min. and, in order to minimize weighing errors and speed the determination, 1 g. fat was dissolved in benzene-methanol and aliquots were pipetted. In addition, the developed color was read in a Coleman 11 Spectrophotometer and peroxide values were calculated from standard regression equations.

[11] Tocopheryl-*o*-quinone and tocopheryl-*p*-quinone.

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FORMATION OF BACTERIAL INHIBITORY ZONES IN WHEY AGAR BY RAW MILK

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SUMMARY

This investigation is concerned with the nature of bacterial inhibitory zones of milk, their incidence, and a technique for measuring the antibacterial property of raw milk. The disc assay test for antibiotics in milk was adaptable for the detection of natural inhibitors in raw milk. By substituting lyophilized milk tablets for the saturated paper discs, the sensitivity of the test was increased. Generally, raw milk produced two clearly defined zones, labelled primary and secondary, respectively, against *Bacillus subtilis*. A primary zone of inhibition first appeared after 12 hr. at 72° F. The secondary zone only appeared after the plates were incubated, refrigerated, and then reincubated. This secondary phenomenon caused disappearance of the bacterial colonies like that associated with lysis. It appears that the presence of the natural inhibitor in milk is independent of the breed of the cow. The initial zone size varied seasonally, and tended to decrease in diameter or disappear during the winter months, whereas secondary zones were less affected seasonally.

Freshly drawn cow's milk often exhibits a germicidal action. As early as 1901, Hunziker *(4)* showed that high-heat treatment of milk destroyed the bactericidal effect, and Chambers (1), in 1920, indicated that its specificity was dependent not only on the individual cow but also on the bacterial species. A literature review covering the period prior to 1927 was presented by Jones *(5)*. In general, the extent of the germicidal effect was measured by direct counting of bacteria or by lactic acid production.

During disc assay studies for antibiotics in milk *(6)*, small zones were noted with antibiotic-free samples. This zoning apparently was associated with some natural antibacterial property of the raw milk. The ability to observe this zoning phenomenon with disc assays stimulated a more complete investigation into the general subject, especially when it was noted *(8)* that the sensitivity of measurements could be significantly increased through concentrating the milk. This paper is concerned with the incidence and nature of the zoning phenomenon in raw milk.

METHODS

Milk samples. Raw milk samples, collected fresh from individual cows of the Cornell University dairy herd, were cooled to 40° F. and tested within 24 hr.

Test organisms. Spore suspensions of *Bacillus subtilis* [1] were of the type recommended for the disc assay procedure. When not obtained commercially,

Received for publication July 29, 1957.

[1] Commercial preparations obtained from Difco Laboratories, Detroit, Michigan. Other strains of *B. subtilis* used were No. 8 and No. 9524.

spores were prepared by the method of Foster and Woodruff *(3)*, using a modification suggested by Silverman *(7)*.

Freeze-drying. A simple apparatus (shown in Figure 1) proved to be an efficient freeze-dryer. A temperature differential was maintained in the desiccator by placing its lower portion in the port of an ice cream cabinet at —18° C.

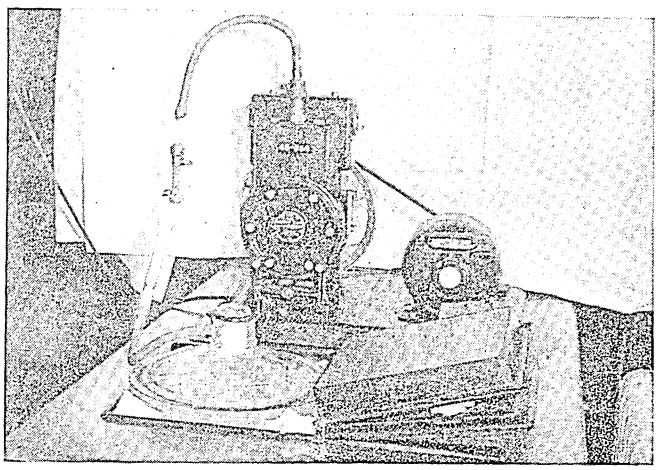


FIG. 1. Apparatus for freeze-drying milk samples.

The upper area was exposed to the atmosphere. The evaporated water condensed and formed a layer of ice on the bottom of the desiccator. This eliminated the need for a condenser between the vacuum tube and desiccator, but a calcium chloride tube was placed in this position as a precautionary measure.

Technique for detecting zones. Milk samples were concentrated to a fine white powder by freeze-drying, usually in less than 12 hr. Samples of this powder were compressed to a 13-mm. diameter circular tablet, weighing 0.1 g., in a Stokes [2] pill-maker. (Another simple way to form tablets is to place the desired weight of powder in a Pyrex-glass powder funnel and compress the powder into a tablet by pounding with a glass rod.)

The milk tablets were placed on the surface of seeded whey agar with a sterile spatula and assayed in a manner similar to that described for the standard paper disc assay method, without inverting the test plates. Resulting zone diameters, illuminated and magnified in a Quebec colony counter, were measured with calipers to the nearest 0.5 mm.

For comparative purposes, the standard disc assay method *(8)*, using 13-mm. paper discs, served as a control.

Lower incubation temperature for a longer period (20° C. for 12 hr.) was found to give slightly larger zones than at 37° C. for 6 hr., recommended in the standard disc assay test, and was more convenient for the analyst. The lower temperature was used throughout this study, but to detect antibiotics added to milk, the higher incubation temperature always is advocated.

[2] Stokes Machine Co., Philadelphia, Pennsylvania.

TABLE 1

Primary inhibitory zone displayed seasonally by raw milks [a]

Cow No.	Zone size																			
	1953		1954										1955							
	7/16 Disc	7/21 Disc	2/12 Disc	2/17 Disc	Tab.	4/22 Disc	5/6 Disc	Tab.	8/5 Disc	Tab.	9/30 Disc	12/28 Disc	Tab.	1/31 Disc	Tab.	2/12 Disc	Tab.	5/8 Disc	6/25 Disc	Tab.	7/18 Disc
	Diameter of zone minus diameter of disc or tablet *(mm.)*																			
1	2	2	**	**	**	0	1	2	1	5	3	<1	6	**	**	**	**	0	0	<1	0
2	4	2	**	**	**	0	1	4	2	5	3	*	*	0	6	0	4	0	0	<1	0
3	2	2	0	*	*	0	1	4	2	3	3	<1	<1	0	0	**	**	2	0	<1	0
4	5	*	0	0	0	**	*	6	3	4	3	<1	6	0	0	0	4	<1	0	5	0
5	*	*	0	0	*	1	1	*	4	*	3	<1	0	*	*	*	*	*	0	6	<1
6	2	2	**	**	*	0	<1	*	3	7	4	<1	6	**	**	**	**	*	0	<1	*
7	*	*	*	*	*	*	*	5	*	*	4	<1	0	0	0	0	0	**	*	*	*
8	*	*	*	*	*	*	*	*	*	*	4	<1	0	0	0	0	4	<1	0	6	1
9	*	*	*	*	*	*	*	*	*	*	3	<1	0	0	0	<1	0	2	0	8	2
10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	3	0	6	0
11	*	*	*	*	*	*	*	*	*	*	*	*	*	4[m]	6[m]	0	4	4	*	6	*
12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	4	0	*	3
13	*	*	*	*	*	*	*	*	*	*	*	*	*	0	0	0	0	*	0	8	*

Legend: 0—no zone; *—not tested; **—animal was dry; m—mastitic milk (Cow No. 11).

Disc—Test was made using 13-mm. diameter paper disc.

Tablet—Test was made using 13-mm. diameter dried raw milk tablet.

Values are averages of duplicate samples.

Cows 1, 6, and 11 are Holsteins; 7, 9, and 13 are Jerseys; and 10 and 12 are Brown Swiss.

[a] Against *Bacillus subtilis* bacteria grown on whey agar.

RESULTS

Effectiveness of technique. Replacement of paper discs with tablets of lyophilized milk showed, with many milks, natural zones where none was evident before. Also, zones previously observed in some raw milks were reproduced with significant increases in area, averaging about tenfold. The sensitivity of detecting bacterial inhibition by raw milks thus was materially improved (Tables 1 and 2).

Character of zoning. The phenomena of natural zoning of milks were more complex than previously had been anticipated. This became apparent inadvertently, when some finished plates displaying zones of inhibition were stored at low temperatures and later reincubated. Another type of zoning, distinct from the first, appeared on these plates.

With milks having the ability to inhibit bacteria, incubation at 20° C. for 12 hr. produced a zone of slight haziness averaging 15 mm. in diameter. This is called the primary zone (Table 1).

TABLE 2

Secondary inhibitory zone displayed seasonally by raw milks[a]

Cow No.	Zone size										
	1954		1955								
	12/28		1/21		2/12		4/24	5/25	6/25		7/18
	Disc	Tablet	Disc	Tablet	Disc	Tablet	Disc	Disc	Disc	Tablet	Disc
	Diameter of zone minus diameter of disc or tablet *(mm.)*										
1	4	14	**	**	**	**	7	0	0	14	0
2	*	*	4	15	3	10	**	0	6	15	5
3	4	15	3	14	**	**	**	0	0	11	0
4	5	15	2	9	0	13	**	0	0	9	0
5	4	19	*	*	*	*	*	1	3	0	*
6	2	15	**	**	**	**	8	0	0	15	2
7	4	17	0	12	0	10	*	**	*	*	*
8	4	19	0	11	0	11	*	2	0	0	0
9	4	19	<1	13	0	12	*	2	0	0	5
10	*	*	*	*	*	*	6	0	0	0	1
11	*	*	8[m]	18[m]	3	15	5	0	*	*	*
12	*	*	*	*	*	*	6	0	0	9	*
13	*	*	0	13	5	11	*	0	4	8	0

Legend: 0—no zone; *—not tested; **—animal was dry; m—mastitic milk.
Disc—Test was made using 13-mm. diameter paper disc.
Tablet—Test was made using 13-mm. diameter dried raw milk tablet.
Values are averages of duplicate samples.
Cows are same as in Table 1, except that No. 5 is a Jersey, instead of a Holstein.
[a] Against *B. subtilis* bacteria grown on whey agar.

Storage of this incubated plate at 5° C. for 24 hr., followed by reincubation at 20° C. for 12 hr., often resulted in another, more vivid, type of zoning (Figure 2). Formation, usually adjacent to the first zone, was apparent within 4 hr. after reincubation. The new circular zone began in an area of live bacterial colonies and worked its way back toward the milk tablet. In 12 hr. it had the ability to pass through the agar to this point, clearing all the haze apparent

in the primary zone. At times, however, it would reach only the periphery of the primary zone, leaving a ghost halo of living bacterial colonies separating two distinct zones, one slightly hazy, the other clear and transparent. This zone, averaging 20-mm. diameter through the center of the milk tablet, is hereinafter referred to as the secondary zone (Table 2). The secondary zone occurred also in milks not displaying the initial zoning.

The primary zone displayed characteristics normally associated with bacterial inhibition, but the secondary zone appeared to be associated with lysis of the test bacteria. Sporulation of bacteria may also account for the disappearance of colonies. However, very few spores were found, microscopically,

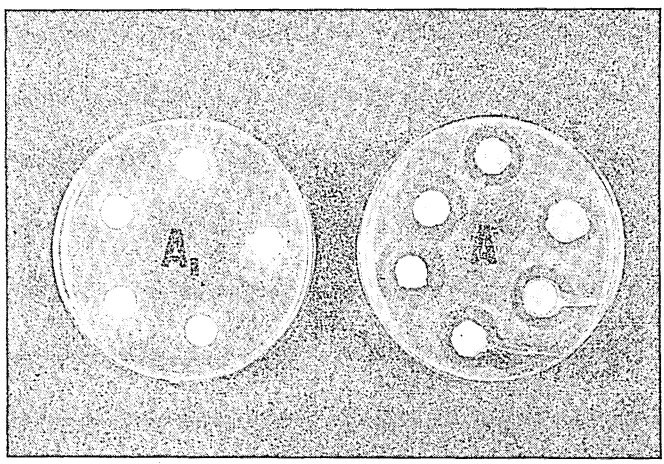


FIG. 2. An example of the secondary-zoning phenomenon encountered in present study. A. Concentrated raw milk tablets on whey agar, using *B. subtilis*. Note clear secondary zones. A_1. Concentrated heated milk tablets on whey agar, using *B. subtilis*. Note in A_1 the absence of primary and secondary zones as a result of heat treatment.

in secondary-zone agar areas stained with malachite green, thus strengthening the concept that lysis is responsible for the secondary zone.

Incidence and size of zoning. Milk from individual cows and from cows of different breeds of the Cornell University herd produced pronounced variations in primary-zone size (Tables 1 and 2). The largest primary zoning, 53 mm. in diameter, resulted from a sample of untreated mastitic milk, whereas winter milk from normal cows gave the smallest zones and lowest incidence. Secondary zoning was subject to much less seasonal variation.

In the winter of 1953, starting in December, no primary zones were obtained from mixed milk of the Cornell herd; none was found until late April of the next year. During the winter of 1954, the cows of the Cornell herd did not exhibit the primary zoning between December and April. When they were allowed outdoors, only in the barnyard, primary zoning was evident. They were not put on pasture until later in the season.

Secondary zoning exhibited less variation between milks of individual cows that did primary zoning. This also applied with milks obtained in different

seasons of the year. The ability of milk to create conditions resulting in primary and secondary zoning was not related to breed of cattle, for all breeds tested showed similar variations.

When pronounced natural inhibitory zoning is encountered in milk, the zone diameter may not express this concentration linearly, because the diffusion rate in agar becomes the limiting factor. Dilution of the raw milks, containing high concentrations before lyophilizing and assaying, restores true linearity.

TABLE 3

Dilution of raw milk with sterile distilled water and the resulting primary inhibitory zones[a]

Raw milk	Water	Raw milks tested		
		A	B	C
(ml.)		Diameter of zone minus diameter of disc *(mm.)*		
10	0	2	4	4
9	1	2	3	3
8	2	0	2	*
7	3	0	2	2
6	4	0	2	1
5	5	0	1	0
4	6	0	0	0
3	7	0	0	0
2	8	0	0	0
1	9	0	0	0
0	10	0	0	0

Values are averages of duplicate samples.

*Not tested.

[a] Against *B. subtilis* bacteria grown on whey agar.

Some milks (Table 3) show considerably more titer of natural inhibitory substances than do others. For example, at the point where the inhibitory effect was at a minimum, one milk required a dilution of nine parts of water to one part of milk; another 1:1, while a third sample was diluted 1.5:1.

DISCUSSION

In the present study, it was observed that some raw milks had the ability to produce two clearly defined types of zoning phenomena, analogous to those of staphylococcal hot-cold lysis, against sensitive bacteria. Only recently, Chevalier *et al. (2)* noted that inhibitory strains of streptococci grown as isolates in agar seeded with sensitive lactobacilli produced a similar type of zoning around each of their separate colonies. Here, then, is an example of raw milk and inhibitory streptococci which display in agar a similar behavior pattern against sensitive bacteria.

Agents such as lysozyme can lyse *B. subtilis* in a manner similar to that displayed by raw milk in the secondary zoning, but for reasons which are elaborated on in detail in a following paper, lysozyme apparently is not the causative agent with respect to milk.

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CHANGES IN THE BACTERIOPHAGE SENSITIVITY OF LACTIC STREPTOCOCCI

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SUMMARY

Twenty-three fast acid–producing pure cultures of lactic streptococci, propagated daily at 22° C. in skimmilk fortified with 0.75% nonfat milk solids, were tested periodically in litmus milk for changes in sensitivity to purified bacteriophage filtrates. The following changes occurred in the 10-mo. test period: *(1)* Six cultures became resistant to their homologous bacteriophages and sensitive to one bacteriophage. *(2)* Three resistant cultures became sensitive to one bacteriophage. *(3)* Six cultures became mixtures and required two bacteriophages to stop acid production. *(4)* Three cultures became resistant to their homologous bacteriophages and then were resistant to a mixture of those available. In all cases studied, except one, the bacteriophages were adsorbed only by sensitive cultures. The adsorption behavior of the bacteriophages, the types of change observed, and the methods used to prevent changes indicate that changes in sensitivity resulted from displacement of sensitive bacterial populations by resistant populations that arose from rare mutant bacteria. The rare mutants had a selective advantage and (in all cases except one) altered bacteriophage-adsorption characteristics (assumed to reflect changes in surface structure).

Luria and Delbruck *(8)* have shown that pure cultures usually contain resistant mutants that give rise to resistant bacterial populations after the cultures are attacked by bacteriophages. Resistant mutants arise independently of bacteriophage action and may differ from sensitive cells by only a simple, gene-controlled difference in surface structure that prevents adsorption of the bacteriophage *(8, 14)*. Complementarity of the surface structures of the bacteriophage and the resistant bacteria may be restored by an independent mutational change in the bacteriophage *(7)*.

The bacteriophage sensitivities and resistances of pure cultures have been considered stable characteristics. Without the advantage resulting from elimination of sensitive organisms by bacteriophage action, resistant mutants usually have little chance in competing against the predominant sensitive cells. Novick and Szilard *(12)* studied sporadic cycles in the numbers of resistant mutants in a culture of *Escherichia coli* in the absence of bacteriophage action. Decreases in proportion of resistant bacteria were ascribed to displacement of the entire bacterial population by a new sensitive population arising from adaptive, sensitive mutants. Increases in proportion of resistant bacteria were ascribed to mutations to resistance in each new sensitive population. Such periodic replacement of a bacterial population by a new sensitive type having selective advantage over the previous type has been substantiated as a mechanism that postpones the establishment of mutational equilibrium *(1)*.

Received for publication August 19, 1957.

METHODS

Culture sources and propagation procedure. Pure cultures of lactic streptococci were isolated from cultures obtained from the Dairy Research Institute of New Zealand, from commercial cultures, and from raw milk. Only cultures that coagulated skimmilk rapidly were studied. These were propagated daily (six days per week) in skimmilk fortified with 0.75% nonfat milk solids and autoclaved at 121° C. for 13 min. The inoculum was five drops of culture per 33 ml. of fortified skimmilk. Inoculated bottles were incubated at 22° C. for 16 hr. and then refrigerated. Skimmilk was examined for inhibitors by a test using *Streptococcus thermophilus* and *Streptococcus cremoris (5)*. Skimmilk that contained inhibitors was not used.

Procedure for determining bacteriophage sensitivity. Tubes of litmus milk inoculated with two drops of the desired bacteriophage filtrates, and one control tube, were inoculated with one drop of fresh culture. The tubes were incubated at 32° C. for about 8 hr. and compared soon after the control tube had coagulated. Bacteriophages that did not permit typical coagulation were considered active.

Procedure for determining adsorption of bacteriophages. A sample of bacteria was grown at 32° C. for 2 hr. in sterile skimmilk. Aliquots of bacteria and bacteriophage filtrate were transferred to a tube of sterile skimmilk that was preheated to 32° C. After 14 min. had been allowed for adsorption, the mixture was centrifuged for 8 min. at 5,700-5,900 r.p.m. in a Servall, Model XL, angle centrifuge and the supernatant assayed immediately for unadsorbed bacteriophage particles. The counts of bacteria present in the adsorption mixtures were from 3.5×10^7 to 1.2×10^8 per milliliter. The bacteriophage counts in the mixtures were from 5.0×10^6 to 2.0×10^7 per milliliter.

Enumeration procedures. Bacteria were enumerated by plate count on modified trypticase soy agar *(9)*. Bacteriophages were enumerated by plaque count *(13)*.

RESULTS

Changes in sensitivity to bacteriophages. Approximately 20 cultures, purified by colony isolation, had been propagated daily for about 4 mo., when it was observed that three originally resistant isolates were now susceptible to Bacteriophage 20a. Finding this, the sensitivities of the cultures were rechecked, disclosing that 11 of the cultures had sensitivities different from those observed initially. During the following 6 mo. the remaining cultures and others isolated from changed cultures were checked weekly. Seven additional changes in sensitivity were observed. Only five cultures, about one-fourth of those studied, did not change. Some of the cultures produced lactic acid slightly more rapidly after they changed.

After the changes had been observed, the activities of the bacteriophages were checked using unchanged cultures. The results revealed that the activities of the bacteriophages had not changed during the course of the experiment.

The cultures used for checking the bacteriophages were some that had been kept frozen and some that were obtained from the original sources.

All of the cultures except ML3 were classified as *S. cremoris*, on the basis of neither growing at 40° C. nor producing ammonia from L-arginine. Culture ML3 was classified as *S. lactis* before and after it became resistant to Bacteriophage m13.

TABLE 1

Summary of the changes observed in the sensitivity of lactic streptococci to bacteriophages

Culture	Source	Bacteriophage sensitivity	
		Before change	After change
4B	Commercial culture	4b	4b[a] + 20a[a, b]
4B	Commercial culture	4b	20a
4B1	4B	4b	4b + ab3
4B2	4B	4b	Resistant
KH2	KH	kh	kh[a] + kha[a]
KH2	KH	kh	Resistant
KH3	KH	kh	kh + 20a[a]
M9B	Commercial culture	kh	kh + ab3
ML3	ML3	m13	Resistant
K1	K[c]	kha	kha[a, d] + e8[a, d]
TR	TR	tr	20a[d]
R1	R1	r1	20a[d]
R6	R6	r6	20a
E8	E8	e8	20a[d]
M9C	Commercial culture	15c	20a[d]
A	Milk	Resistant	20a
B	Milk	Resistant	20a
C	Milk	Resistant	20a

[a] Cultures sensitive to this bacteriophage were isolated from the changed culture.

[b] Bacteriophage 20a was isolated from cottage-cheese whey, using a culture of *Streptococcus cremoris* that had been isolated from a commercial culture. This culture was discarded and Bacteriophage 20a was purified and thereafter propagated on Culture 20A, which had been isolated from milk.

[c] This culture, labeled K, was received from a commercial source. Upon arrival it was a mixture of bacteria, some sensitive to Bacteriophage e8 and some sensitive to kha.

[d] Enumeration of this bacteriophage by the limiting active dilution method with the changed culture gave the same result as enumeration with the homologous host.

The changes in sensitivity that were observed are listed (Table 1). They can be divided into the following four types:

1. In six of the 18 changes, cultures became resistant to their homologous bacteriophages. Simultaneously, resistance to Bacteriophage 20a changed to sensitivity. Four of these cultures were lysed by a filtrate of Bacteriophage 20a when it was diluted $1:10^9$ with litmus milk, indicating that the activity of the bacteriophage was multiplication rather than mere inhibition *(2, 10, 17)*. The other two cultures were not used in checking activity of the diluted filtrate.

2. In three of the changes, cultures that had been resistant to all of the available bacteriophages became sensitive to Bacteriophage 20a. These changes were similar to those of the first type, except for the original resistance of these cultures.

3. In the third type of change, single-sensitivity bacteria became a mixture with two sensitivities, requiring two bacteriophages to stop acid production.

Both types of bacteria could be isolated. Six of the 18 changes were of this type. The difference from the first type might have been merely that the less competitive bacteria had not yet been overgrown. Two examples follow:

(a) Culture 4B. Some activity was indicated for Bacteriophage 4b by the fact that Culture 4B coagulated weakly when inoculated with the bacteriophage. The weakly coagulated culture was plated and two colonies isolated. These were resistant to Bacteriophage 4b and susceptible to 20a. We then found that a mixture of Bacteriophages 4b and 20a lysed Culture 4B.

Culture 4B (not inoculated with bacteriophage) was plated and 12 isolates were found susceptible to Bacteriophage 4b. Three were retained. After 5 wk. one of these cultures changed. A mixture of Bacteriophages 4b and ab3 was then required to stop acid production.

(b) Culture KH. Three cultures were checked for changes in susceptibility to Bacteriophage kh. KH2 and KH3 had been isolated from the original culture; M9B had been isolated from a commercial culture.

KH2 was now questionably lysed by the unpurified mixture of wheys, and appeared resistant to Bacteriophage kh. This culture was plated and an isolate chosen that was sensitive to the mixture of wheys. This isolate, KHA, was used in isolating an active bacteriophage from the mixture of wheys. Since the resulting bacteriophage only partially lysed KH2, ten resistant cultures were isolated by inoculating KH2 with the bacteriophage and then permitting coagulation and plating the coagulum. All of these resistant cultures were lysed by the original kh phage; none was lysed by the new bacteriophage. It was thereafter determined that a mixture of Bacteriophages kh and kha lysed Culture KH2.

KH3 now was only questionably lysed by kh. Two resistant isolates from this culture, prepared by plating KH3 after coagulation in the presence of kh, were resistant to Bacteriophages kh and kha; they were sensitive to 20a. A mixture of kh and 20a stopped the production of acid by KH3.

M9B remained sensitive to Bacteriophage kh until 2 mo. after the first recheck. It then appeared resistant and required a mixture of Bacteriophages kh and ab3 to stop the production of acid.

4. The fourth type of change occurred in three cultures. These cultures became resistant to their homologous bacteriophages and then were resistant to a mixture containing all the available bacteriophages. The difference from the first type may have been only that bacteriophages active against the resistant cultures were not available.

Methods of avoiding changes in sensitivity. In rechecking sensitivities of the cultures at weekly intervals, it was observed that cultures propagated every other day changed less frequently than cultures propagated daily and that any changes occurred gradually, after they were detectable, over a period of several days to 3 or 4 wk. Cultures plated when they first appeared resistant to their homologous bacteriophages usually yielded sensitive isolates.

Two methods were used to eliminate the need for periodic isolations. The first involved maintaining an extra isolate or propagation of each culture. When one of the cultures developed resistance to the homologous bacteriophage, it was discarded and two propagations were started from the other culture. The second method involved maintaining quantities of lyophilized cultures under refrigeration. Fresh cultures could be started when changes occurred in those being propagated.

Adsorption studies. Adsorption experiments were conducted with several of the cultures and bacteriophages. The results (Table 2) indicate that alterations in adsorption characteristics (assumed to reflect changes in surface structure) occurred simultaneously with changes in sensitivity. The active homologous bacteriophage was readily adsorbed to the cells of a sensitive culture. When a culture became resistant, that bacteriophage was no longer adsorbed. When a culture became sensitive, the bacteriophage that was then active was readily adsorbed, though it had not been before the change.

TABLE 2

Differences in the adsorption of bacteriophages before and after changes in sensitivity in cultures of lactic streptococci

Culture	Source	Sensitivity	Bacteriophage added	Per cent adsorption
KH2S	KH2	kh	kh	97
KH2R	KH2	Resistant	kh	0
ML3	ML3	m13	m13	95
ML3R	ML3	Resistant	m13	0
K1S	K1	kha	kha	86
K1R	K1	e8	kha	0
K1S	K1	kha	e8	0
K1R	K1	e8	e8	97
20A	Milk	20a or c1	c1[a]	93
M9C	Commercial culture	15e	c1	0
M9CR	M9C	20a or c1	c1	90
R1	R1	r1[b]	r1	93
R1R	R1	20a or c1[b]	r1	91
R1	R1	r1	c1	39
R1R	R1	20a or c1	c1	48

[a] Bacteriophage c1 rather than 20a was chosen for adsorption studies because 20a did not produce countable plaques.

[b] Bacteriophage 20a or c1 could inhibit R1; Bacteriophage r1 could inhibit R1R.

One exceptional case—in Cultures R1 and R1R—involved little or no change in the adsorption of bacteriophages. Before a change in sensitivity, Bacteriophage c1 or 20a (which acted the same) inhibited Culture R1; after the change, Bacteriophage r1 inhibited the changed culture. Yet neither of these infections resulted in bacteriophage multiplication. Other reports have described inhibition without bacteriophage multiplication *(2, 10, 17)* and slow adsorption with bacteriophage multiplication *(4)*.

DISCUSSION

The results, together with the findings of Luria and Delbruck *(8)*, Novick and Szilard *(12)*, and Atwood *et al. (1)*, suggest an explanation of the changes

in sensitivity that occurred in the absence of bacteriophage action. Resistant mutants *(8)* and adaptive mutants *(1, 12)* normally occur as independent mutations in separate bacterial cells in the predominant (sensitive) component of a bacterial population. This does not rule out the possibility that there may be an occasional adaptive mutant with altered surface structure. Such a mutant would be expected to develop into a resistant population that displaces the sensitive population. That such mutations did occur in these cultures of lactic streptococci is indicated by the types of change observed, the methods that prevented changes in sensitivity, and the adsorption behavior of the bacteriophages.

A possibility that cross contamination of cultures, followed by growth and domination, could result in changes similar to those observed has been considered. The following points indicate that the above explanation is more tenable: *(a)* Cross contamination is less likely than contamination with bacteriophages, particularly in a laboratory where bacteriophages are cultured. There were no sudden failures in acid production, indicating that the cultures were adequately protected against bacteriophage contamination. *(b)* Changes occurred most frequently in two very fast acid–producing cultures, KH and 4B. *(c)* Culture R1 adsorbed Bacteriophage r1 readily and c1 slowly, before and after the change in sensitivity. *(d)* The only *S. lactis* culture, ML3, was classified as *S. lactis* after the change in sensitivity. *(e)* Culture C2 (obtained after the above experiments were completed) changed so as to exhibit resistance toward only one of three originally homologous bacteriophages. *(f)* None of the cultures in the study before the isolation of KHA was sensitive to Bacteriophage kha.

Altered adsorption characteristics were apparently not involved in a change in Culture R1 that enabled multiplication of Bacteriophage c1 (or 20a) and prevented multiplication of Bacteriophage r1. This case serves to emphasize that bacteriophage multiplication does not always result when particles of a bacteriophage are adsorbed to the cells of a culture. However, it appears that, in most cases, the factor preventing bacteriophage multiplication is adsorption failure.

Cultures of lactic streptococci that are not known to have been acted on by bacteriophages—though bacteriophages active against them are often known—generally are referred to as primary growth; whereas, cultures that are known to have arisen after lysis of sensitive cells by a bacteriophage are referred to as secondary, or resistant, bacteria. Secondary bacteria have often been found to be sensitive to bacteriophages other than those used in their isolation *(11, 16, 18)*. In the present study, where resistance apparently was gained by natural selection of mutants, several of the resulting cultures were sensitive to other bacteriophages. This suggests that there is not a significant difference between primary and secondary, except as the terms might apply to the relationship between a culture and a particular bacteriophage—in which case the term sensitive or resistant will usually suffice.

Differences between a resistant population naturally selected and one artificially selected (by lysis of sensitive cells) can be the result of one or both of two differences between the mechanisms involved. Without bacteriophage

action, a rare resistant mutant must have some other competitive advantage—at least a small one—to be able to displace a sensitive population. On the other hand, when the sensitive cells in a culture are lysed, a resistant culture may arise from any viable resistant bacterium, regardless of competitive ability. In the former case, the resulting cultures might be more stable. Secondly, when a bacteriophage is employed in the isolation of resistant cultures, there is a possibility that the resulting cultures will be lysogenic *(15)* or carriers of the bacteriophage *(6)*.

Hunter *(6)* suggested that the constant action in nature of a variety of bacteriophages on lactic streptococci accounts for some of the differences in sensitivity. On the other hand, the present results suggest that, when cultures multiply for long periods in the absence of bacteriophage action, differences in sensitivity tend to disappear as a result of natural selection. This could account for the comparatively few different bacterial strains or types (as judged by difference in bacteriophage sensitivity) that were found in an examination of 14 commercially used cultures *(3)*.

ACKNOWLEDGMENT

Appreciation is expressed to Dr. H. R. Whitehead for contributing cultures and bacteriophages.

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CONTINUOUS AND AUTOMATIC PROPAGATION OF DAIRY CULTURES[1]

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SUMMARY

A small fermentation system was devised for continuous automatic propagation of dairy cultures. The fermentor is a circulatory, 1-in. diameter, stainless steel pipe assembly of 1,000-ml. capacity, in which skimmilk is continuously and slowly circulated. Acidity is controlled with a pH meter and meter-relay which activates a solenoid valve controlling the skimmilk inflow. The fermentation system has been operated for 12 wk., but not without contamination and undesirable fementations. Successful trials free from contamination have been conducted for 3 wk. These have provided information regarding the throughput rate at various temperatures between 50 and 90° F. At 90° F. the retention time or growth rate was 30 min. The critical pH level was 5.3, below which the rate of growth was reduced. Dairy cultures can be propagated continuously at maximum growth rates without impairment of flavor or activity, with constant temperature and pH (above 5.3 for maximum rate) and in continuous motion.

Many industrial fermentation processes today are operated continuously, automatically. Continuous fermentations have been investigated and applied, with varying degrees of success, to the production of antibiotics, enzymes, acetic acid, casein, fatty acids and their esters, lactic acid, industrial alcohol, bakers' and food yeast, solvents, sulfur, and vinegar. When continuous fermentations are applied successfully to commercial operations, the advantages obtained are space and labor savings, as well as quality improvement and greater yield of the product.

Reviews *(11, 16)*, theoretical treatments *(4, 7)*, and other publications on various related techniques and processes *(1, 2, 6, 8, 10)* are of value in obtaining an understanding of the techniques and current research in this rapidly growing field.

In the field of dairy science, commercially successful, continuous fermentation processes are used in the production of lactic acid and in the manufacture of commercial casein *(15, 18)*. For lactic acid production, Whittier and Rogers *(17)* maintained cultures of lactobacilli and streptococci at 43° C. between pH 5.0 and 5.8 by periodic additions of calcium carbonate to fermenting whey. Kempe *et al. (5)* and Finn *et al. (3)* have reported results of studies of continuously controlled pH on lactic acid production by *Lactobacillus delbruckii* at various levels between 3.5 and 6.0. Meyer *(9)* reported successful automatic cream neutralization for butter manufacture, using electronic pH control.

The purpose of this study was to assemble the equipment needed for suitable control of continuous fermentations, and to investigate the fundamental bacteriological principles involved during continuous growth or propagation of lactic

Received for publication June 6, 1957.

[1] Florida Agricultural Experiment Station's Journal Series, No. 625.

acid–producing dairy cultures, used in the manufacture of buttermilk and various kinds of cheeses. It was believed that the information obtained would be of value in the development and application of continuous, automatic methods of processing the various fermented dairy products.[2]

EQUIPMENT AND APPARATUS

To aid others who might be interested in further work along these lines, a detailed description of the apparatus and equipment used is given.

Figure 1 schematically illustrates the apparatus design and controls. The internal capacity of the circulatory pipe system was 1,000 ml.

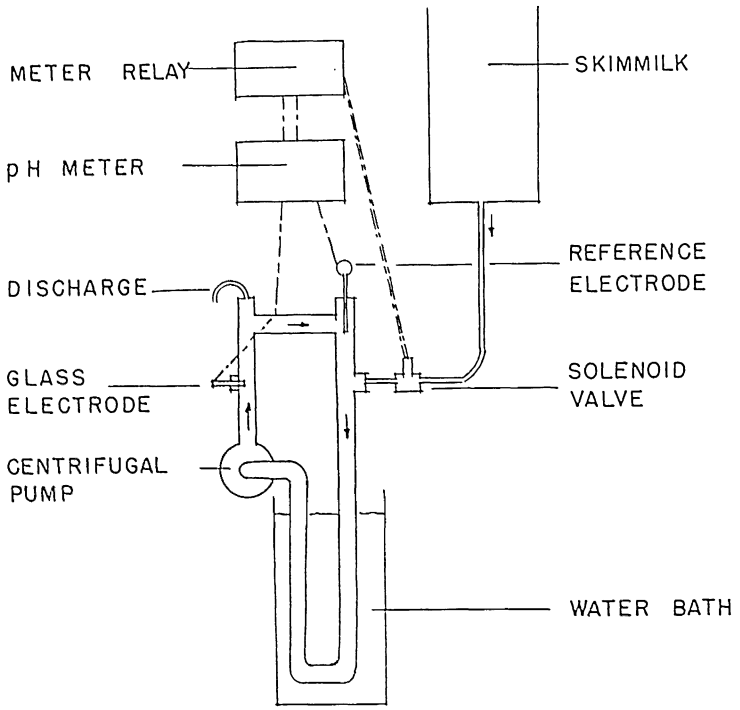


FIG. 1. Schematic illustration of fermentation system and controls.

A Beckman Model H-2 pH meter was used. The manufacturer recommends that this model not be used in grounded electrode systems such as flow lines, but reasonably satisfactory results were obtained under the conditions described here. The various components were grounded as required. General purpose glass electrodes (Model 8990-80) were used. Sleeve-type reference electrodes (Model No. 8970-14) were found to give better results than those of the fiber junction type.

[2] Recently another paper has appeared on this subject, W. J. HARPER AND D. A. SEIBERLING, Method and Apparatus for the Continuous Production of Cheese-Forming Curd. U. S. Patent 2,781,269. *J. Dairy Sci.*, 40: A30, 1957.

The meter-relay was obtained from Assembly Products, Inc., Chesterland, Ohio. It is known as a single-contact, automatic-reset Versatrol, Catalog No. 3581, with Model 451-C single-contact high limit. Its range is 0 to 1 milliampere DC, with a 2-sec. interval timer. Using the pH meter reading, which was synchronized with the scale on the meter-relay, it was possible to select a desired pH and manually set the contacts at the desired pH on the meter-relay for automatic pH control. The output of the meter-relay operated at 110-volt, ¼-in. solenoid valve on and off as required to maintain constant pH.

The circulation pump was a Tri-Clover 1-in. milk pump (Type MS—No. 2075, patented January 27, 1931), which was salvaged for this study. The impeller was replaced with a new blade, approximately 1½ in. long, which reduced the rate of flow to 2½ gal. per minute at 1,725 r.p.m.

The entire assembly was made with standard 1-in. stainless steel milk piping and fittings. It was so designed that the entire system—including the pump—could be autoclaved after assembly. The electrodes were sterilized by alcohol immersion and aseptically attached after autoclaving the system. This procedure has its limitations and might have been a source of contamination. The saturated KCl was not sterilized and also may have been a source of contamination.

When in operation, the immersion of part of the fermentation pipe system into a water bath equipped with heater, stirrer, and thermostat was satisfactory for accurate temperature control.

METHODS

For total bacteria counts, the regular standard plate count procedures were used with Plate Count Agar (Difco), M-P-H Medium (BBL), or Tomato Juice Agar (Difco). For coliform counts, violet red bile agar medium was used.

The creatine test was used to aid in evaluating flavor quality, but in general it was found that organoleptic examination by qualified judges was the most satisfactory manner of judging the quality of the product.

All skimmilk used was reconstituted to give a final serum solids concentration of 10.5 %. The skimmilk was sterilized by autoclaving in quart glass containers approximately three-fourths full for 16 min. at 15 lb. of pressure. The sterile milk was then aseptically transferred to the skimmilk storage tank of the fermentation system and held at 40° F.

PROCEDURE

All trials were conducted using regular commercial lactic streptococci dairy starter cultures. In some instances, the fermentation system was inoculated with fresh liquid cultures which had been propagated in the usual manner. In other instances, the fermentation system was inoculated by simply pouring in powdered cultures.

When in operation, the skimmilk supply tank was located at a higher point than the discharge outlet of the fermentation system. This permitted the circulator system to remain completely full. Upon actuation of the solenoid valve, the fermentation discharged by overflowing an amount of product equal to that

permitted to flow into the system. The inflow continued until the pH level was raised approximately 0.2 to 0.3 unit.

It was possible to cause the input to become relatively uniform by throttling the flow through the rubber hose with a screw clamp, but difficulties then were encountered due to occasional plugging of the system with particles of coagulated milk. The most successful operation was obtained when the flow was adjusted to permit about 150 to 200 ml. of skimmilk to enter at one time over a period of a few minutes and then to stop for from 10 to 30 min., depending upon the throughput rate and temperature of operation.

RESULTS

The fermentation system has been operated continuously for periods of up to 12 wk., but not without some difficulties of contamination and undesirable fermentations. Approximtely one-half of all trials failed—owing to contamination within the first 24 hr.—even when performed with the greatest of care. Considerable improvement of techniques or apparatus is needed for the system to prove of practical value. However, it is significant that some trials were successful, which is the first evidence to that effect. Successful trials (free from contamination) of from 2 to 3 wk. duration have been conducted. These have provided a better understanding of the cultural and biochemical characteristics, under conditions of continuous growth and in constant slow motion or agitation by circulation in the circulatory pipe system.

During these studies, all acidities were determined by both titration and pH. The relationship of titratable acidities and pH is shown (Figure 2). There was always good agreement of the two methods. The solids content was maintained at a constant level of 10.5%.

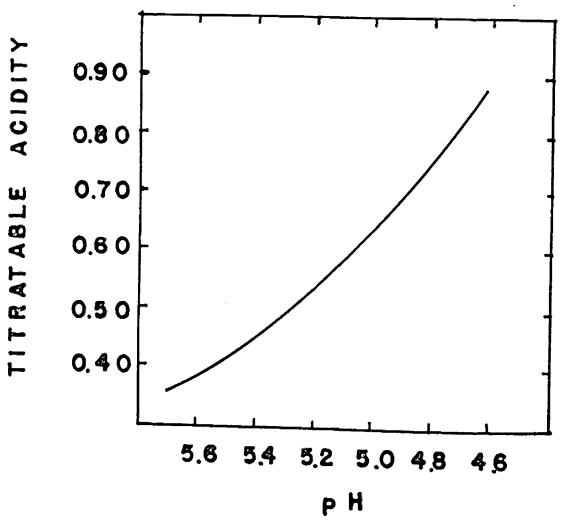


FIG. 2. Relationship of pH and titratable acidity in reconstituted nonfat milk solids at 10.5% concentration.

An important consideration of a continuous fermentation system is the throughput rate, or the rate of flow per volume of fermentor. The holdup, or retention, time is calculated as one over the throughput rate per hour. For all practical purposes, the retention time as used here may be considered equal to the growth rate, or generation time, of the microorganisms present.

When the fermentor was set at a given pH and constant temperature and allowed to flow at the rate influenced by these factors, the total count remained reasonably constant at the level indicated. Total microorganism plate counts at the various pH levels are shown (Figure 3).

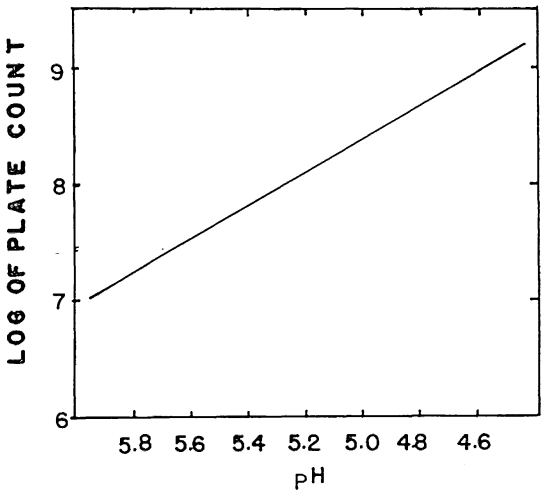


FIG. 3. Influence of pH on the total plate counts.

Figure 4 shows the retention time at various temperatures between 50 and 90° F. at pH 5.5. The curve shows that, as the temperature increased, the retention time decreased significantly. This indicated an increase in the growth rate of the organisms present.

Influence of pH on the retention time is shown (Figure 5). As the acidity increased, the presence of greater amounts of lactic acid tended to reduce the rate of organism growth, although the total count was higher (as shown in Figure 3). There also was greater reduction in rate at the lower temperature of 68 than at 80° F. The reason for this difference was not apparent, but may have been simply individual strain differences between the two cultures used in obtaining these data.

DISCUSSION

The generation time at 90° F. for the dairy starter cultures used was 30 min. Comparing this with other data, Powell *(14)* showed that the generation time of individual bacteria varied widely. He reported generation times of from 6 to 50 min. for *Streptococcus faecalis*. Politi *(13)* also observed "that considerable differences can be had from one strain to another, especially in an advanced stage of growth." He reported that the maximum multiplication rate for *Strep-*

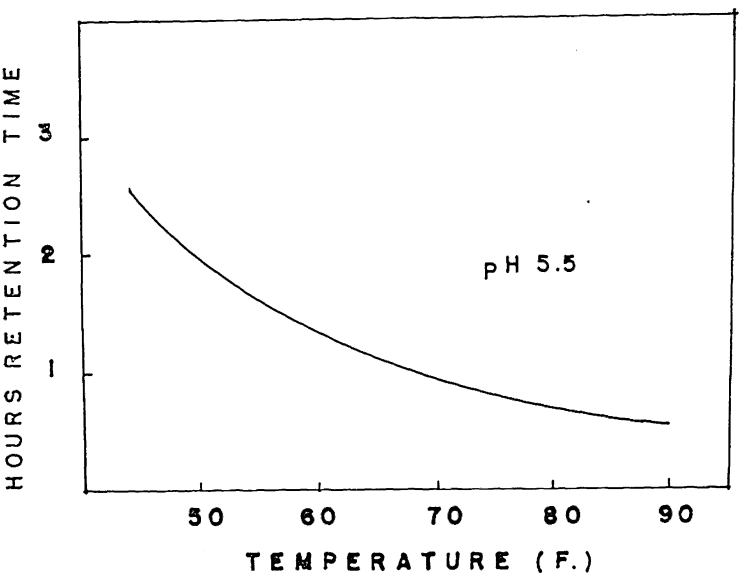


FIG. 4. Influence of temperature on the retention time of the cultures.

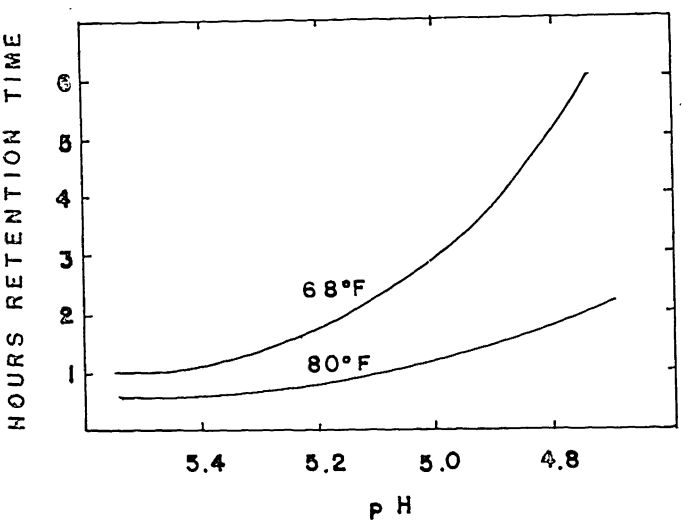


FIG. 5. Influence of acidity (pH) on the retention time of the cultures.

tococcus lactis at 34° C. was 30 min. Herbert *et al. (4)* reported that the doubling time of *Aerobacter cloacae* was 49 min. The generation times of the starter cultures reported here are in general agreement with other organisms. This showed there was no significant difference in growth rate under conditions of slow agitation and continuous propagation.

The limiting pH level seems to be somewhat higher under conditions of continuous growth than in single-growth cycle batches. Overcast *(12)* showed that pH 5.0 was almost the limiting level for several strains of *S. lactis* used in an excellent study of the influence of pH on the proliferation of lactic streptococcus bacteriophage. In all of the trials reported here, the first measurable indication of a reduction in growth took place at pH 5.3, with greater reductions as the pH was lowered. Longsworth and MacInnes *(6)* and Kempe *et al. (5)* showed that careful control of pH at the optimum of 5.8 to 6.0 resulted in more complete fermentation at faster rates in the production of lactic acid by *Lactobacillus acidophilus.*

As the pH level lowered, the first signs of visible coagulation occurred at pH 5.0 to 4.9. The maintenance of satisfactory body and texture characteristics is essential in dairy products, if not in starters. When the fermenting skimmilk was removed from the fermentor above pH 5.3 and allowed to remain motionless, normal coagulation occurred. Preliminary evidence indicates that such coagulation can take place while the products is in very slow motion through a stationary pipe system.

The conditions under which suitable strain balance occurs in mixed cultures have not been established. When mixed cultures were used, the desired aroma slowly disappeared over a period of from seven to ten days, as measured by organoleptic tests. At the beginning, the flavor and aroma were excellent, but they progressively decreased until only a clean acid flavor was detectable. In other trials, with single-strain lactic streptococcus cultures, the flavor remained uniformly satisfactory until contamination occurred. When contamination occurred, off-flavors resulted, as well as reduction in starter activity.

It appears that the application of continuous fermentation to commercial dairy operations would be of considerable value and has future possibilities. Based on the information available, it is possible to speculate that continuous automatic methods of processing the various dairy products will prove feasible. This work does establish certain fundamentals and shows the need for further investigation.

ACKNOWLEDGMENTS

We deeply appreciate the technical assistance of Orlean Clarke and Dudley Thomas.

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ELECTRONIC MICROSCOPIC OBSERVATIONS OF THE STRUCTURE OF CHEESE

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SUMMARY

This study was initiated to develop a method for the examination of the ultra-fine structure of cheese with the electron microscope. It was used to study the structure of Cheddar cheese made from variously treated milk. Freshly broken surfaces of chilled Cheddar cheese were covered with a parlodion film. Next, the film was stripped and the replica side covered with a formvar film. After dissolving the parlodion portion, the formvar film was shadow-cast with gold and examined under the electron microscope.

Observations made on replica films showed specific surface patterns for cheese made from raw, pasteurized, and pasteurized homogenized milk.

At least three groups of workers *(3, 4, 7)* have reported on the use of the electron microscope in the direct examination of ultra-thin slices of cheese. Two of the reports *(3, 4)* show no illustrations; whereas, the third report *(7)* shows illustrations that are difficult to interpret. According to Leber *(4)*, the slicing of cheese into sections of 0.3 μ or less, and the transfer of these ultra-thin slices to a film or grid, are some of the problems in using this method.

Another method, investigated by Imhof and Hostettler *(3)*, is the replica, or indirect, method. Difficulties in obtaining a replica film that would strip properly caused them to abandon this method.

This paper deals with the development of a two-step replica method *(1)* and its use in the indirect examination of the ultra-fine structure of cheese made from raw, pasteurized, and pasteurized, homogenized milk.

EXPERIMENTAL PROCEDURE

Representative portions of cheese were cut with a wire cutter into strips of approximately 5 by 5 by 50 mm. These strips were grouped, wrapped in tinfoil, and chilled for 4 hr. or longer in the ice compartment of a refrigerator. The chilled strips were carefully broken by hand and suitable sections selected for further work. Broken surfaces that were relatively level, and without areas originating from openings within the cheese, were covered immediately with a thin layer of chilled parlodion solution, kept in ice water. The solution consisted of 2 g. parlodion dissolved in a mixture of 0.05 ml. amyl acetate, 20 ml. absolute ethanol, and 80 ml. ethyl ether. The composition of this solution was developed in the early part of this study. It contained a minimum of ethanol necessary to dissolve the parlodion in ether. The small amount of amyl acetate

Received for publication August 1, 1957.

served to prevent curling of the stripped film. Higher concentrations of amyl acetate caused strong adhesion of film to cheese and made stripping difficult or impossible.

After depositing the parlodion solution with an eye dropper, in quantities sufficient to cover the selected surface, the section was placed on clean paper in a manner to permit drainage of excess fluid. As soon as the film showed signs of dryness, a second application of parlodion solution was made. This was repeated five times, or until a film of sufficient thickness and strength was obtained that could be stripped without tearing or breaking. The film, while still on the cheese, was trimmed to size with a sharp scalpel. The trimmed film was stripped and placed in chloroform with replica-side up. This step was introduced to wash off loose cheese particles, as well as to remove any possible fat adhering to the film.

The washed film was placed on a clean glass slide with replica-side up and covered with a solution of 0.125% formvar in chloroform *(1)*. As soon as the formvar had set, the film was transferred to an electron microscope grid with the formvar-side down and immersed into a 1:1 ethanol:ether mixture, to dissolve the parlodion film. This required about 15 min. Next, the grid and formvar film with the replica-side up were removed from the solvent, air-dried, gold-shadowed in a Radio Corporation of America shadow-caster Model EMV-6 at an angle of 15°, and examined under a Radio Corporation of America electron microscope Model EMU-2D.

Pictures were taken of the most commonly observed types of replica structure of cheese on a given film, or several films, obtained from the same lot of cheese. Best pictures were obtained at magnifications of from 5,000 to 10,000.

RESULTS

The cheese examined in this study was taken from experimental lots made in connection with another project. The 6-mo.-old Cheddar cheese examined in 1955 was made from unstandardized Holstein milk. Additional series of 6-mo.-old cheese made from Holstein and standardized Jersey milk were examined in 1956. All cheese was made by the same method *(5)*.

More than 200 replica films were examined under the electron microscope and a total of 75 pictures taken. A representative series of photographs, obtained from replica films of Holstein milk cheese in 1955, is shown (Figure 1). In general, pictures taken in 1956 from Holstein and Jersey milk cheese were rather similar in pattern to those shown in Figure 1.

A close examination reveals certain differences in the surface patterns of the four types of cheese. The differences shown were observed repeatedly, and are regarded as differences in the ultra-fine structure of cheese made from variously treated milks. The raw-milk cheese *(a)* shows a surface of volcanic appearance, with long fibers extending to a considerable height above the general surface of the cheese. Such fibers are not evident in the other three types of cheese. A possible explanation for this difference may be the more

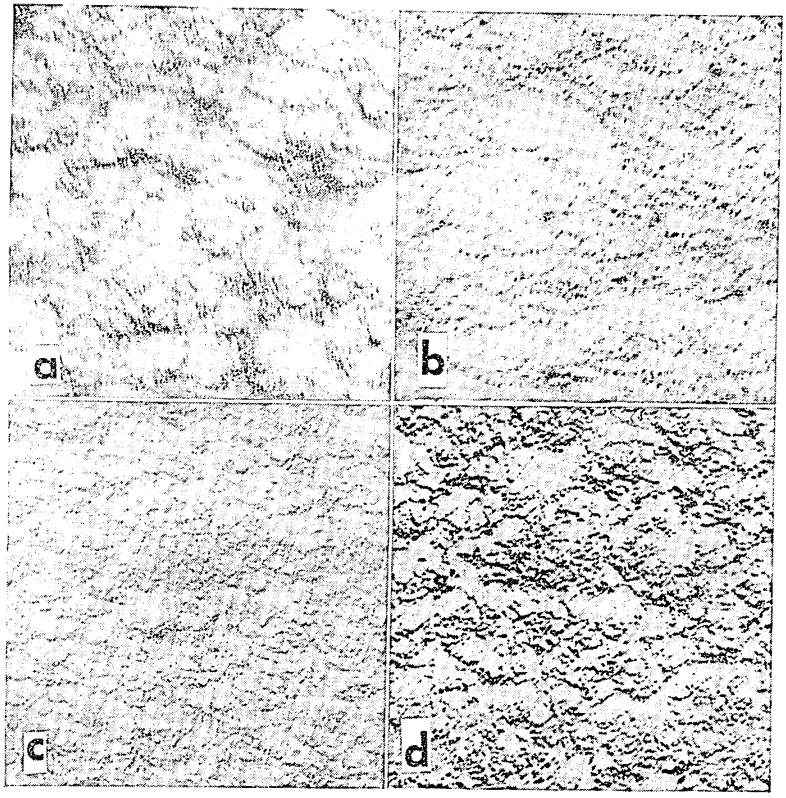


FIG. 1. Electron microscope photographs of surface replica of a series of Holstein milk cheese made from *(a)* raw, *(b)* pasteurized, *(c)* pasteurized, homogenized at 1,000 p.s.i., and *(d)* pasteurized, homogenized at 2,000 p.s.i. Magnification 21,000 ×.

elastic body observed in raw-milk cheese. The pasteurized milk cheese *(b)* differs in surface patterns from the two pasteurized, homogenized milk cheeses *(c, d)*. This may be owing to the fact that homogenization changes the physical structure of casein *(2)* and progressively reduces the elastic properties of cheese *(5)*. That homogenization must be considered an important factor is evidenced by the greater difference in surface patterns between pictures *(b)* and *(d)* than between *(b)* and *(c)*, picture *(c)* representing cheese made from milk homogenized at 1,000 p.s.i. and *(d)* at 2,000 p.s.i.

DISCUSSION

Considering the limitations of the electron microscope, in that only ultrathin preparations lend themselves to examination in a vacuum under the bombardment of electrons, it seems that a replica method, such as developed in this study, has considerable merit. Although Imhof and Hostettler *(3)* have published a method recently by which defatted cheese, hardened with ethanol and formaldehyde, may be embedded and cut successfully into slices of 0.1 μ or less, the method is more involved and at best shows the structure of cheese only after

treatment with several reagents. Since Imhof and Hostettler did not show any pictures, no comparison is possible at this time.

In breaking the chilled cheese by hand, it was reasoned that the break would occur in all instances at the weakest points in the area under stress. Breaking the cheese would also eliminate such distortion of the broken surface pattern as might take place by mechanical cutting. However, no comparison was made to prove or disprove the above assumption.

The application of chilled parlodion solution on chilled cheese was used to produce the minimum interaction between the solvents and the cheese constituents, thereby obtaining as true a replica as possible of the freshly broken cheese surface. The gradual build-up of the parlodion film, as the first replica, was essential in obtaining a film that would dry uniformly on the surface of the cheese and produce the most nearly identical replica.

Studies conducted thus far have been with aged Cheddar cheese only. The above method may prove useful in observing possible structural changes of curd during ripening. Other possible applications are comparison studies of structures of acid- and rennet-type curd of natural and reconstituted milk curd, and many others.

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EFFECTS OF REDUCING RENNET AND ADDING CALCIUM CHLORIDE ON THE MANUFACTURE AND CURING OF CHEDDAR CHEESE[1,2]

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SUMMARY

This study showed the lack of real benefits derived from using low levels of rennet with added calcium chloride in the manufacture of Cheddar cheese.

Cheese was made from raw and pasteurized milk during four seasons. Each lot of cheese consisted of three vats to which 90, 45, and 45 ml. rennet per 1,000 lb. of milk was added, respectively. The third vat of milk was further treated with 0.02% calcium chloride. The cheese was cured at 50° F. for 36 wk. Using subnormal amounts of rennet delayed curd formation in both raw and pasteurized milk. Adding 0.02% calcium chloride with one-half normal amounts of rennet gave normal curd formation and yield, but protein breakdown was slow, and curdiness persisted longer in the finished cheese. The quality of the cured cheese was not influenced by variations in the rennet and calcium chloride used, but persistence of curdiness was undesirable in young cheese made with reduced levels of rennet, especially when supplemented with calcium chloride.

Adding calcium chloride to milk for cheese-making reduces the amount of rennet needed for optimum curd formation *(12, 17)*, improves curd-making properties under certain conditions *(29)*, and may increase the yield of cheese *(14, 17, 21)*. The use of calcium chloride with less rennet has been advocated by some practical operators for reducing costs. The Federal Definitions and Standards of Identity *(8)* permit the addition of the equivalent of 0.02% anhydrous purified calcium chloride to milk for cheese-making.

It is the purpose of this paper to report the results of using reduced amounts of rennet, with and without calcium chloride, on the manufacture and ripening of Cheddar cheese made from raw and pasteurized milk. The factors observed were: curd firmness at cutting time; recovery of milk solids in the cheese; extent of ripening in terms of proteolysis and fat hydrolysis at 8 and 36 wk. of age, and cheese quality at 2, 8, and 36 wk. after making.

EXPERIMENTAL METHODS

Cheese-making. The milk came from the mixed milk supply of the University of Wisconsin dairy manufacturing laboratory.

Cheese was made in September, December, March, and July, to represent milk from four seasons. Four lots of cheese were made in each of these months:

Received for publication August 19, 1957.

[1] Published with the approval of the Director, Wisconsin Agricultural Experiment Station, Madison.

[2] This study was supported in part by research grants from Marschall Dairy Laboratories, Madison, Wisconsin, and Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin.

two lots from pasteurized milk and two from raw milk. One extra lot of raw-milk cheese was made in September. Raw milk was made into cheese on the day it was received. Milk which had been pasteurized at 143° F. for 30 min. and cooled to 40° F. was held overnight before manufacturing. Each lot consisted of three vats in which equal amounts of identical milk and culture were used. Vats A, B, and C received 90, 45, and 45 ml. of rennet, respectively, per 1,000 lb. of milk. Calcium chloride was added at the rate of 0.02% of the milk to Vat C. These vats will be referred to in this discussion as Treatments A, B, and C. Fresh commercial rennet and calcium chloride of analytical reagent grade were used. Calcium chloride to be added to the milk was dissolved in distilled water at the rate of 90.8 g. anhydrous calcium chloride per liter. The interval between adding rennet and cutting the curd was standardized at 30 min. for all vats, in order to measure the clotting effects of each treatment.

All vats of milk were made into cheese by a routine procedure *(22)*. After milling, the curd was sprayed with approximately 25% of its weight of water at 90° F., and was forked intermittently and completely drained before salting. The cheese was formed into 10-lb. blocks, wrapped in Parakote, and cured at 50° F. for 36 wk.

Fat and dry matter. Fat in milk was measured by the Babcock method, and casein was estimated by formol titration *(9)*. Milk solids were measured by drying 2 g. of milk in 50-ml. beakers containing clean, dry sand. Samples were dried at 110° C. for 16 hr. in a forced-draft oven. Whey was sampled at dipping and tested for fat by the American Association method *(10)*, and for solids by drying 2 g. in covered aluminum dishes for 16 hr. at 110° C. in a forced-draft oven. Cheese solids were measured by drying 2 g. in 50-ml. beakers at 110° C. for 16 hr. *(23)*.

Curd firmness. After rennet had been added and mixed with the milk, duplicate 400-ml. beakers were filled from each vat. Each beaker was suspended in the vat in such a way that the surface of the milk in the beaker was at or below the surface of the milk in the vat. At the time of cutting, 30 min. after adding rennet, curd firmness was measured on the curd in the two beakers by using the official curd knife *(6)* and the apparatus of Sommer and Matsen *(27)*.

Measurement of ripening. Rate of protein decomposition in the cheese was estimated at 8 and 36 wk. by measuring total nitrogen in the cheese, and the per cent of total nitrogen soluble in a water solution of sodium citrate at pH 4.7. All tests were made in duplicate. A modification of Sommer's *(26)* method was used for extracting soluble nitrogen. A sample of cheese weighing 25 g. was placed in a Waring Blendor jar with 5 g. of C.P. sodium citrate and 170 ml. of distilled water, and mixed at high speed for 15 min. The mixture was quantitatively transferred to a 600-ml. beaker, acidified to pH 4.7 with 2 *N* acetic acid, and heated to 50° C. in a water bath. Meanwhile, a filter mat was prepared in a Buchner funnel. Two sheets of Reeve Angel No. 202 filter paper formed the bottom of the mat. A mixture of J. M. Celite and water was poured on the filter paper and compacted with suction. Two more sheets of filter paper were placed over the Celite and the entire mat rinsed with distilled water. The

sample at 50° C. was filtered through the prepared Buchner funnel with gentle suction. The residue was washed twice with small amounts of distilled water at 50° C. and refiltered after each washing. The combined filtrates were then made to a volume of 500 ml. Nitrogen in the cheese and in the acidified sodium citrate extract was measured by the Kjeldahl-Wilfarth-Gunning method *(2)* with the boric acid modification *(18)*.

An estimate of free fatty acids in the cheese at 8 and 36 wk. of age was made by a method developed by Kielsmeier and Swanson *(13)*.

Cheese quality. Each cheese was scored at 2, 8, and 36 wk. of age by three judges who worked independently. Their individual values for each cheese were averaged to give the score of that cheese. The scoring was based on points ranging from 1 to 6, in which 1 was excellent; 2, desirable; 3, satisfactory; 4, objectionable; 5, very objectionable, and 6, unsalable. The judges commonly used one-fourth of a point to distinguish differences.

The first changes in body breakdown were estimated by the curdiness of the cheese, which the judges were asked to consider as a factor separate from body score. This was evaluated by using: 0, indicating no curdiness; 1, slightly curdy; 2, distinctly curdy; 3, very curdy, and 4, extremely curdy.

Cheese flavor intensity, as distinguished from flavor score, was rated by the judges as: 0, possessing no cheese flavor; 1, slight cheese flavor; 2, distinct cheese flavor; 3, pronounced cheese flavor, and 4, extreme cheese flavor.

Statistical treatment of data. Analyses of variance, as outlined by Cochran and Cox *(5)* for randomized blocks, were made on all the data. The significance of differences between treatments in each analysis of variance was determined by the multiple range test described by Duncan *(7)*. Differences were considered statistically significant at the 95% level and highly significant at the 99% level, unless otherwise indicated. Confidence limits at the 95% level of significance were calculated for each mean value presented in the tables. These limits were calculated according to a procedure described by Snedecor *(25)*. Unless otherwise stated, differences or effects of treatments are noted only when they have been found to be statistically significant.

RESULTS

Milk. Average percentages of fat, casein, and total solids in the milk are given (Table 1). Values are normal for this geographical area and indicate a comparable composition of raw and pasteurized milks used.

TABLE 1

Average percentages[a] of fat, casein, and total solids in the milk

	Lots	Pasteurized milk	Lots	Raw milk
	(No.)	*(%)*	*(No.)*	*(%)*
Fat	8	3.53 ± .07	9	3.58 ± .09
Casein	8	2.39 ± .08	9	2.45 ± .09
Total solids	8	11.57 ± .27	9	11.69 ± .27

[a] Means and 95% confidence limits.

Curd firmness at cutting. The data (Table 2) show curd firmness measurements made 30 min. after adding rennet. Reducing the amount of rennet decreased curd firmness, but the addition of 0.02% $CaCl_2$ restored the curd firmness characteristic of normal rennet levels. The low curd firmness values

TABLE 2

Effect of reduced quantities of rennet with and without added calcium chloride on curd firmness at time of cutting

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Curd firmness[a]	Lots	Curd firmness[a]
	(ml.)	*(%)*	*(No.)*	*(g.)*	*(No.)*	*(g.)*
A	90	0.00	8	12.0 ± .9	9	15.6 ± .7
B	45	0.00	8	9.8 ± .9	9	12.0 ± .7
C	45	0.02	8	13.0 ± .9	9	15.0 ± .7

[a] Means and 95% confidence limits.

reported (Table 2) for pasteurized milk show the probable influence of pasteurization, and of the cool aging *(1, 24)* which occurred during overnight storage of this milk.

Yield. The data in Table 3 indicate a marked reduction in recovery of milk solids in raw-milk cheese when one-half the normal amount of rennet was

TABLE 3

Effect of rennet and calcium chloride on the proportion of milk solids retained in the cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Milk solids in cheese[a]	Lots	Milk solids in cheese[a]
	(ml.)	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
A	90	0.00	6	50.09 ± .35	9	50.92 ± .30
B	45	0.00	6	49.97 ± .35	9	50.30 ± .30
C	45	0.02	6	50.37 ± .35	9	51.05 ± .30

[a] Means and 95% confidence limits.

used. Yield efficiency was restored by the use of 0.02% calcium chloride in Treatment C. No differences in yield were attributable to Treatments A, B, or C on pasteurized-milk cheese.

Measurement of fat in the whey at dipping showed significantly greater losses in raw-milk whey only following the use of half-normal amounts of rennet. Percentages of fat in whey from raw milk following Treatments A, B, and C were 0.36, 0.39, and 0.35%, respectively, and for pasteurized milk, 0.36, 0.36, and 0.33%, respectively.

Solids-not-fat content of raw wheys were all alike at 6.10%, whereas whey from pasteurized milk following Treatments A, B, and C contained 6.07, 6.09, and 6.10%, respectively.

The explanation of differences in recovery of milk solids observed is suggested by the data (Table 2). Those lots which showed low curd-firmness values tended to have lower recovery of solids in the cheese. The effects of milk composition and cool aging on the yield of cheese solids were not measured, but the combined data in Tables 1, 2, and 3 suggest that lower average casein content and cool aging might have been responsible for soft curd formation which resulted in lower yields in all three treatments of pasteurized milk.

Ripening. Protein decomposition during ripening was significantly affected by the rennet treatments. The data (Table 4) show that at 8 and 36 wk.,

TABLE 4

Effect of rennet and calcium chloride on the soluble nitrogen and extractable fatty acids in cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese			Raw-milk cheese		
			Lots	Soluble nitrogen[a]	Fatty acids[b]	Lots	Soluble nitrogen[a]	Fatty acids[b]
	(ml.)	*(%)*	*(No.)*	*(8 weeks)*		*(No.)*	*(8 weeks)*	
A	90	0.00	8	14.73 ± .34	3.53 ± .09	9	16.47 ± .36	4.03 ± .12
B	45	0.00	8	12.16 ± .34	3.59 ± .09	9	14.22 ± .36	4.08 ± .12
C	45	0.02	8	12.69 ± .34	3.50 ± .09	9	14.32 ± .36	3.99 ± .12
				(36 weeks)			*(36 weeks)*	
A	90	0.00	8	27.21 ± .84	3.59 ± .09	9	30.62 ± .47	5.27 ± .14
B	45	0.00	8	23.91 ± .84	3.80 ± .09	9	27.30 ± .47	5.04 ± .14
C	45	0.02	8	24.10 ± .84	3.58 ± .09	9	27.73 ± .47	5.10 ± .14

[a] Soluble nitrogen as per cent of total nitrogen, showing mean values and 95% confidence limits.

[b] Ml. of 0.05 *N* alcoholic KOH required to neutralize extractable fatty acids from 5 g. of cheese. Mean values and 95% confidence limits are shown.

protein decomposition in the control cheese, Treatment A, was greater than that in cheese made by either Treatments B or C. The difference in soluble nitrogen between the control and experimental cheese, while marked at 8 wk. of curing, was even greater after 36 wk. Amounts of soluble nitrogen in cheese made by Treatments B and C were alike at 8, and again at 36 wk. of age. Proteolytic changes showed the same trends in both pasteurized- and raw-milk cheese. These findings agreed with those of Babel *(3)*.

The conclusion is obvious that the initial changes in normal ripening are definitely associated with rennet action. It seems probable that these initial changes in the protein are associated with the development of smoothness of body. This will be shown by the observations of curdiness (Table 6). Whether these changes in protein are measurably important in flavor development is highly questionable, in view of the flavor scores of the cheese (shown in Table 5).

The fatty acids liberated during curing were not affected by the levels of rennet or by the calcium chloride used.

The data of Table 4, however, show that greater amounts of extractable fatty acids were produced in the raw-milk cheese, which also had the highest flavor intensity. This would be expected from the results of studies by several other workers who, generally, are agreed that rates of fatty-acid liberation are associated with flavor development (*4, 15, 16, 28*).

It is not surprising, in the light of the fatty-acid data (Table 4), to find that flavor scores and intensity of cheese flavor were not affected by experimental treatments.

Cheese quality. Table 5 presents the body and flavor scores of the cheese at 2, 8, and 36 wk. of age.

TABLE 5

Effect of rennet and calcium chloride on average body and flavor scores of cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Lots	Pasteurized-milk cheese		Lots	Raw-milk cheese	
				Flavor score[1]	Body score[1]		Flavor score[1]	Body score[1]
	ml.	*%*	*No.*	*2 weeks*		*No.*	*2 weeks*	
A	57	0.00	8	2.53 ± .14	2.59 ± .18	9	3.10 ± .15	2.71 ± .19
B	45	0.00	8	2.65 ± .14	2.71 ± .18	9	2.98 ± .15	2.88 ± .19
C	45	0.05	8	2.69 ± .14	2.72 ± .18	9	3.04 ± .15	3.14 ± .19
				8 weeks			*8 weeks*	
A	90	0.00	8	2.69 ± .11	2.38 ± .17	9	2.80 ± .25	2.47 ± .21
B	45	0.00	8	2.83 ± .11	2.69 ± .17	9	2.88 ± .25	2.68 ± .21
C	45	0.05	8	2.84 ± .11	2.48 ± .17	9	2.94 ± .25	2.70 ± .21
				36 weeks			*36 weeks*	
A	90	0.00	8	2.69 ± .28	2.35 ± .17	9	3.17 ± .28	2.58 ± .28
B	45	0.00	8	2.88 ± .28	2.38 ± .17	9	3.06 ± .28	2.68 ± .28
C	45	0.05	8	2.75 ± .28	2.38 ± .17	9	3.24 ± .28	2.68 ± .28

[1] Mean scores and 95% confidence limits. 1—excellent; 2—desirable; 3—satisfactory; 4—objectionable; 5—very objectionable, and 6—unsalable.

The levels of rennet and calcium chloride used had no significant influence either on the flavor score or on the total score of pasteurized- or raw-milk cheese at any age.

The body scores of cheese made by Treatments B and C were slightly inferior at 2 and 8 wk. of age to those of the control cheese, made by Treatment A. These slight differences, however, were not always statistically significant.

Openness was the most common body criticism of the young cheese. The extent of openness at 2 and 8 wk. is shown in Table 6.

Openness in young cheese was exaggerated by using low levels of rennet. The data in Table 6 show this effect clearly. Adding calcium chloride made the effect more pronounced. These trends were still evident, but to a lesser degree,

at 8 wk. of age. Some of the judges classified lack of ideal body at 8 wk. as evidence of persistent curdiness. There was no curdiness in any of the cheese at 36 wk. The greater curdiness at 2 wk. in the cheese made by Treatments B and C could be an important economic factor in the cheese industry, since most cheese is graded when it is from 5 to 14 days old. However, it is extremely unlikely that these differences would be commercially important beyond 8 wk., in cheeses cured like these, at 50° F.

TABLE 6

Effect of rennet and calcium chloride on average degree of curdiness in cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Curdiness[a]	Lots	Curdiness[a]
	(ml.)	*(%)*	*(No.)*	*(2 weeks)*	*(No.)*	*(2 weeks)*
A	90	0.00	8	1.38 ± .27	9	1.78 ± .28
B	45	0.00	8	1.75 ± .27	9	2.36 ± .28
C	45	0.02	8	1.90 ± .27	9	2.50 ± .28
				(8 weeks)		*(8 weeks)*
A	90	0.00	8	0.19 ± .31	9	0.22 ± .34
B	45	0.00	8	0.56 ± .31	9	0.61 ± .34
C	45	0.02	8	0.66 ± .31	9	0.56 ± .34

[a] Means and 95% confidence limits. 0—no curdiness; 1—slightly curdy; 2—distinctly curdy; 3—very curdy, and 4—extremely curdy.

Flavor intensity scores at 2, 8, and 36 wk. showed that a reduction in the amount of rennet, with or without the addition of calcium chloride, had no significant influence on intensity of cheese flavor.

DISCUSSION

The roles played by rennet enzymes and calcium ions in milk-clotting have never been completely defined. However, it has been demonstrated that there are two general phases to the clotting of milk by rennet. An alteration of the casein by rennet enzymes is followed by gel formation, which occurs only in the presence of calcium ions.

Calcium ions by themselves are known to have an aggregating effect on casein micelles *(19, 20, 30)*, and it appears that rennet makes the casein even more sensitive to aggregation by calcium. Therefore, aggregation of casein micelles might be considered as partial clot formation. Thus, the addition of calcium chloride to milk would cause some casein aggregation and require less rennet action to produce a visible clot and a firm curd. Another factor which, no doubt, plays some part in the calcium chloride effect is the influence of this salt in lowering the pH of the milk, thus increasing the curd-shrinking action of the rennet enzymes. These combined effects upon moisture in the curd can actually delay loss of curdiness in normal ripening periods.

Rennet action continues beyond the stage of coagulation and is measurable as percentage of total cheese nitrogen soluble in an acidified sodium citrate solution. When this action is decreased in cheese-making, by using reduced amounts of rennet (Table 4), curdiness persists in the young cheese. Adding calcium chloride and reducing the amount of rennet, establishes two conditions favoring persistence of curdiness.

Curdiness is normally associated with young cheese, but would be criticized at 2 wk. of age if it were excessive, or if the judge expected such curdiness to persist unduly. Curdiness did persist in the cheese made with reduced amounts of rennet and especially in that which contained added calcium chloride.

Hobson and Schaars *(11)* found that the age of mild cheese in grocery stores varied from 4 to 8 wk. At this age, the curdiness found in the experimental cheese made with reduced amounts of rennet, along with added calcium chloride, might still detract from its market value. This same cheese cured for 36 wk. would not be subject to such penalties.

The curing temperature of 50° F. used in these experiments is lower than that commonly used for forced curing of cheese, but higher than the temperatures used for commercial storage. Higher temperatures, if used, would have hastened proteolysis, loss of curdiness, and changes in fat; lower temperatures would have delayed such ripening effects. These facts must be considered in applying the findings of this study to other curing conditions.

This study demonstrated that it was possible to reduce the amount of rennet from 90 to 45 ml. per 1,000 lb. of milk and still obtain satisfactory curd formation in the same time by adding 0.02% calcium chloride to the milk. In making 10,000 lb. of milk into cheese in this way there would be a saving of 15 oz. of rennet, valued at $0.64, based on a price of $5.50 per gallon. It would be necessary to replace that amount of rennet with the equivalent of 2 lb. of purified anhydrous calcium chloride. At $0.24 per pound for a satisfactory grade of calcium chloride, the cost would be $0.48, and the net saving would be only $0.16. If the calcium chloride is purchased in solution as a proprietary preparation, its cost per pound on an anhydrous basis approximates $0.50 at current prices.

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STUDIES RELATED TO THE MANUFACTURE OF SWISS-TYPE CHEESE. I. USE OF HOMOGENIZED MILK

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SUMMARY

Jersey milk standardized to the desired fat:casein ratio with natural or reconstituted skimmilk was used in the manufacture of Swiss-type cheese. All milk was pasteurized at 143° F. for 30 min. In some series, portions of the same milk were homogenized at 0 (control), 500, 1,000, and 2,000 p.s.i. In other series, a blended milk composed of equal parts of 0 and 2,000 p.s.i. treated milk replaced the milk homogenized at 500 or 1,000 p.s.i. Highest yields of cheese were obtained from milk homogenized at 500 p.s.i., followed by blended milk, and milk homogenized at 1,000 p.s.i. Lowest and similar yields were obtained from milk homogenized at 0 and 2,000 p.s.i. A comparison of the quality of the 3-mo.-old cheese showed that only the blended milk cheese excelled the control cheese.

Homogenization of milk was found to lower fat losses in whey as well as to decrease fat leakage in cheese.

Previous workers *(2, 5)* have shown that Swiss-type cheese of legal composition *(3)* can be made from pasteurized milk. Although there exist no published data on the use of homogenized milk in the manufacture of Swiss-type cheese, the work of Matheson *et al. (6)* permits speculation that such may be possible. Homogenization of milk is known to reduce the size of fat globules and has been found to lower fat losses in the whey of Cheddar cheese *(8)*.

This study deals with an evaluation of homogenization as a part of the process for making Swiss-type cheese. For comparative purposes, the cheese was made from pasteurized milk and from pasteurized milk homogenized to various degrees. Adjustment of the fat:casein ratio was accomplished with natural or reconstituted nonfat dry milk.

EXPERIMENTAL METHODS

Jersey milk from the College herd testing 4.8–5.6% fat was secured in quantities sufficient to prepare four 200-lb. lots of standardized processed milk. The raw whole milk in each series was standardized with skimmilk of its kind or reconstituted skimmilk [9 lb. U.S. Estra spray-dried, low-heat nonfat dry milk (N.D.M.) + 91 lb. tap water] to a calculated fat:casein ration of 1:0.8, by using van Slyke's formula *(9)*.

Milk in each series was pasteurized at 143° F. for 30 min. It was cooled in the vat to 110° F., and consecutive portions of 200 lb. each were passed through a Manton-Gaulin homogenizer at given pressures on the way to the surface cooler. All milk was cooled to 40° F. or below, collected in 10-gal. cans, refrigerated overnight, and made into cheese the following morning.

The cheese was made in four identical 50-gal., stainless steel Damrow cheese vats. The same make-schedule was rigidly adhered to in all instances.

Received for publication August 22, 1957.

After adjusting the milk to 90° F., 1% active commercial lactic starter was added, followed by the addition of 0.02% $CaCl_2$ dissolved in water. A culture of *Propionobacterium shermanii,* grown in yeast extract for seven days at 85° F., was then added at the rate of 40 ml. per 200 lb. milk. After 60 min. of ripening, the milk was set with commercial rennet at the rate of 90 ml. per 1,000 lb. The curd was cut 30 min. later, using ¼-in. curd knives. Each curd was cut once horizontally and twice each way vertically. Twenty minutes after cutting the curd, one-third of the vat contents was removed in the form of whey. Tap water at 160° F. was added, until the vat contents reached a temperatuer of 120° F.

The curd was agitated and cooked at 102° F. for 60 min. The curd was allowed to settle, pushed to the upper end of the vat, and allowed to mat for 20 min. while draining the whey. The partially matted curd was cut into blocks of suitable size to fill a hoop of the American favorite style. Further treatment of curd and cheese until after salting was identical to that described by Babel and Hammer *(2).*

The salted cheese was paraffined after the rind was sufficiently dry, and ripened at 70° F. for from 2 to 3 wk. until eyes of desirable size had formed. Further ripening took place at from 50 to 60° F. The cheese was judged when 3 mo. old.

Fat tests on milk, whey, and cheese were made by the Babcock Method *(4).* Moisture and salt of the 4-wk.-old cheese were determined by an official method *(1),* whereas the per cent oiling off in cheese was determined as described by Peters *(8).*

All cheeses were scored by three judges according to a score-card (shown in Table 1), using the criticisms listed by Nelson and Trout *(7).* The identity of the samples was withheld from the judges in all instances.

TABLE 1

Score-card used in judging Swiss-type cheese

Items considered	Minimum points per item allowed for Swiss-type cheese classified as				Maximum points per item
	Excellent	Good	Fair	Poor	
Flavor	32	29	26	25	40
Holes and appearance	22	19	16	15	25
Texture	18	16	13	12	20
Salt	10	9	8	8	10
Finish	5	4	3	2	5
Total	87	77	66	62	100

RESULTS

Homogenizing milk at increased pressure resulted in decreases in the elasticity of the curd, at cutting time. It also resulted in slower whey expulsion during cooking, reduced color intensity of cheese, and decreases in fat loss in whey. The lower fat losses in whey gave rise to higher per cent fat in the dry matter, as shown in the data (Table 2).

Progressive increases in homogenization pressure decreased the per cent oiling off in cheese. In general, values of from 15 to 20% were judged as most

TABLE 2
Data on composition and yield of Swiss-type cheese

Milk homogenized at p.s.i.	Total lots involved	Average value per item studied: Whey	Cheese			
		Fat	Oiling-off[c]	Fat in T.S.	Moisture	Yield as % of 0 p.s.i.
		(%)				
0	13	0.58	31.5	47.45	39.10	100
500	5	0.32(.50)[b]	19.6 (30.4)	48.84(47.32)	40.01(39.20)	104.4
1,000	8	0.25(.46)	13.5 (31.4)	49.00(47.80)	39.86(39.10)	103.3
2,000	10	0.27(.62)	6.0 (31.4)	49.25(47.30)	38.90(38.92)	100.0
0 + 2,000[a]	5	0.36(.45)	8.7 (32.6)	49.32(48.44)	40.70(39.40)	103.9

[a] 1:1 ratio of 0 p.s.i. to 2,000 p.s.i. milk (referred to as blended milk).
[b] Values obtained on same series with 0 p.s.i. milk.
[c] Based on grams of free solidified fat obtained from 20 g. of ground cheese.

desirable from the standpoint of mouth feel. Whereas cheese made from milk homogenized at 500 or 1,000 p.s.i., or from blended milk, was higher in moisture than was the control cheese, this was not the case with cheese made from milk homogenized at 2,000 p.s.i.

Average yields were the same for cheese made from 0 to 2,000 p.s.i. treated milk. These lots, while similar in per cent moisture, differed in the per cent fat in total solids. Although total solids in whey were not determined, there appeared to be a larger loss of fine curd in whey from milk homogenized at 2,000 p.s.i. than from the control milk. Yields of the other three types of cheese exceeded those of the control cheese. The higher yields were due mainly to higher per cent moisture and higher per cent fat in total solids in cheese.

Data on the quality of 3-mo.-old Swiss-type cheese are presented (Table 3).

TABLE 3
Data on quality of 3-mo.-old Swiss-type cheese

Milk homogenized at p.s.i.	Total lots involved	Average scores per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
0	13	29.2	18.3	16.5	79.0
500	5	29.0(29.8)[b]	18.0(18.7)	16.3(17.0)	78.3(80.5)
1,000	8	28.5(29.0)	17.7(18.0)	16.5(16.3)	77.7(78.3)
2,000	10	28.1(29.2)	16.0(18.3)	14.0(16.5)	73.1(79.0)
0 + 2,000[a]	5	28.8(28.4)	18.3(17.3)	16.3(15.7)	78.4(76.4)

[a] 1:1 ratio of 0 p.s.i. to 2,000 p.s.i. milk (referred to as blended milk).
[b] Scores obtained on same series with 0 p.s.i. milk cheese.
[c] No criticism on salt and finish.

In general, the quality of the cheese decreased with progressive increases in homogenization pressures. Only the blended milk cheese excelled the control cheese in total score, including flavor, holes, appearance, and texture. Specific flavor criticisms associated more frequently with a particular type of cheese were bitter for control cheese, and unnatural for cheese made from milk homogenized at 2,000 p.s.i. The latter type of cheese was criticized also for

such defects as blind, glass, blow holes, and sponge. Criticisms involving all five types of cheese, listed in the order of decreasing frequency, were lacking flavor, picks, small eyes, and bloat. Fewest criticisms were received by cheese made from milk homogenized at 500 p.s.i. and from blended milk; whereas, most criticisms were received by cheese made from milk homogenized at 2,000 p.s.i.

Standardization of milk with either natural or reconstituted skimmilk resulted in cheese of similar composition and quality.

DISCUSSION

The manufacture of Swiss-type cheese from homogenized milk offers some advantages as well as disadvantages. Lower fat losses in whey permit narrowing of the fat:casein ratio, thereby permitting the maufacture of more pounds of cheese per pound of fat. This increases yield. The reduction of fat globules in milk to a smaller, more uniform size decreases the tendency of creaming during setting, thereby resulting in a more uniform distribution of fat throughout the curd and in a cheese with a richer and more mellow flavor. Decreased oiling-off of fat may also be regarded as an advantage, especially if cheese is exposed for any length of time to higher than safe temperatures, be it during manufacture, ripening, merchandising, or in the home.

In contrast to the advantages listed, progressive increases in homogenization pressure reduced the quality of the ripened cheese. Because of the decrease in the elasticity of the curd, it may be that a modification of manufacture and ripening of the cheese would improve the quality without losing any of the advantages listed. One of the more obvious points of attack would be on the ripening of such cheese, particularly the propionic fermentation.

ACKNOWLEDGMENT

The authors are indebted to F. E. Potter for assisting in the judging of the cheese.

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STUDIES RELATED TO THE MANUFACTURE OF SWISS-TYPE CHEESE. II. FACTORS INFLUENCING RIPENING

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SUMMARY

The ripening of Swiss-type cheese, made from standardized pasteurized Jersey milk, passed through the homogenizer at 0 and 2,000 p.s.i., was studied. Varying the amount of propionic culture inocula revealed that in order to obtain cheese of equal quality, a smaller inoculum should be used with milk homogenized at 2,000 than that unhomogenized. The quality of the ripened cheese was not improved by the addition to the milk of KCl or $MnCl_2 \cdot 2H_2O$; the addition of a culture of *Lactobacillus casei* improved it significantly. When cheese made from unhomogenized milk was salted in brine containing KCl, some beneficial effect was observed; this was not the case when milk was homogenized at 2,000 p.s.i.

Pasteurization of milk has been reported to delay the ripening of Swiss cheese *(3, 12)*. Ripening of cheese was enhanced by adding to pasteurized milk, cultures of *Lactobacillus casei*, *p*-amino benzoic acid, and $MnSO_4$ *(3)*. Both Mn^{++} and K^+ have been found beneficial for the growth of *Lactobacillus casei (2, 5)*.

Conditions most favorable for propionic fermentation in Swiss cheese have been reported as temperatures of 72–75° F. *(4)*, pH 5.2–6.8 *(4, 9, 11)*, and an initial population of 100 propionic bacteria per gram of cheese *(4)*. The formation of eyes, or holes, of the desired size, shape, and number is favored by an elastic curd *(4, 9)*, free from mechanical openings and controlled rate of fermentation *(9, 11)*. Since ripening usually results in a more inelastic curd, eye formation should precede the proteolytic breakdown of cheese *(4, 11)*. Excessive propionic fermentation results in cheese with a tough, rubbery body *(7)*, and also prevents or retards the proteolytic breakdown of the curd *(4)*. Free proline *(12)*, calcium and sodium propionate *(1)*, have been reported as being responsible for the desirable sweet flavor in Swiss cheese.

The present study deals with the bacteriological aspects of the ripening of Swiss-type cheese made from pasteurized, and pasteurized, homogenized milk.

EXPERIMENTAL PROCEDURE

Methods other than those described in an earlier paper *(10)* are listed below.

Forty-hour cultures of *L. casei*, grown in peptonized milk broth, were added to milk prior to renneting at the rate of 40 ml. per 200 lb. Potassium chloride and $MnCl_2 \cdot 2H_2O$ were added at the rate of 40 g. and 3 mg., respectively, per 200 lb. of milk. The rate of added KCl to a 23% brine was 200 mg. per liter. Total volatile acid in ripened cheese was determined by the method of Lane and Hammer *(8)*.

Received for publication August 22, 1957.

TABLE 1

The influence of amount of propionic culture inoculum upon the quality of 3-mo.-old Swiss-type cheese

Amount of inoculum (ml/200 lb. milk)	Trial	Score per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
20	1[a]	27	20	17	79
40		26	17	13	71
0	2[a]	25	17	15	72
10		26	18	15	74
20		31	21	18	85
40		28	16	13	72
0	3[b]	28	18	17	78
5		28	19	19	81
10		Atypical—discarded			
20		29	19	19	82

[a] Nonhomogenized milk.
[b] Milk homogenized at 2,000 p.s.i.
[c] No criticism on salt and finish.

RESULTS

The influence of amount of propionic culture inoculum upon the quality of 3-mo.-old Swiss-type cheese is shown in the data (Table 1). Inocula of 20 ml. produced cheese superior in flavor, holes, appearance, and texture to cheese containing 40 ml. inoculum, as shown in Trials 1 and 2 with nonhomogenized milk cheese. In Trial 2, inocula of 0 and 10 ml. produced cheese inferior in quality to that containing 20 ml. inoculum. Determination of total volatile acids from cheese of Trial 2 yielded values of 38, 72, 87, and 160 ml. 0.1 *N* acid equivalent per 200 g. of cheese. A similar determination on a lot of 3-mo.-old commercial cheese (not shown), made with 40 ml. inoculum, was found to have a value of 228 ml. of 0.1 *N* acid equivalent. This commercial lot showed excellent eye formation, but was excessive in propionic fermentation and had a tough, rubbery body. Ripening for an additional 7 mo. did not improve the flavor and body of this cheese.

Cheese in Trial 3, made from milk homogenized at 2,000 p.s.i. with 5 to 20 ml. inocula, was similar in quality and superior to the control cheese made without added propionic culture. Cheese made with 10 ml. inoculum was atypical for reasons unknown. Figure 1 shows a photograph of the three lots of cheese from Trial 3.

Swiss-type cheese made from nonhomogenized milk without and with added K^+ and Mn^{++} resulted in cheese similar in quality, as shown (data in Table 2). Other cheese of the same series, containing added culture of *L. casei* showed superior flavor, holes, appearance, and texture as compared with the control cheese. Cheese made with additions of both salts and *L. casei* was superior to the control cheese, but somewhat inferior to the cheese containing added *L. casei* only. The difference in score between cheese of Trial 1 and Trial 2 was due to the different rate of propionic inoculum used. Cheese in Trial 2 with 40 ml.

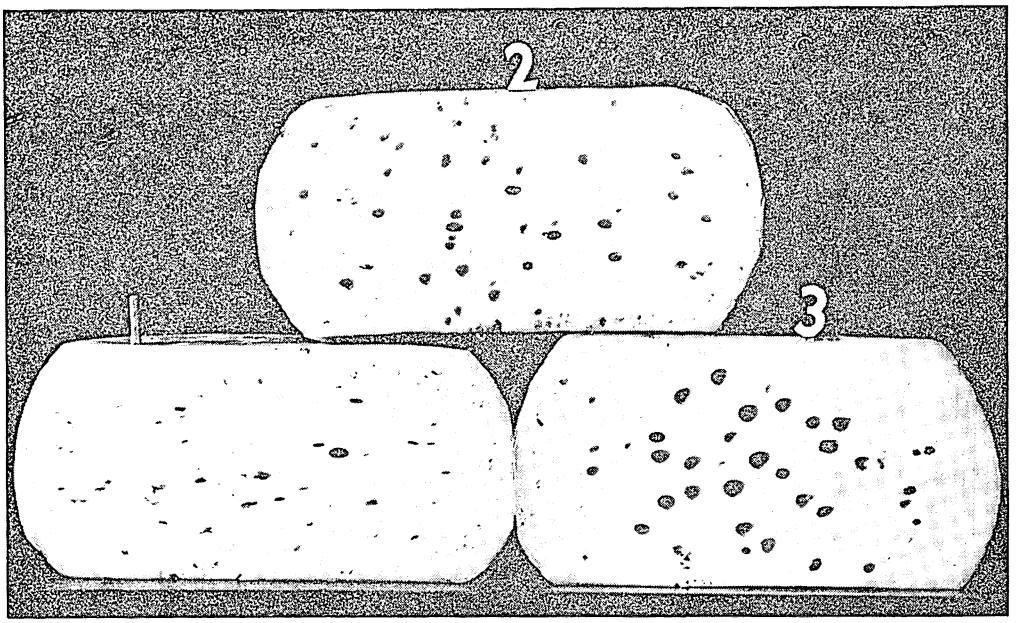


FIG. 1. Swiss-type cheese made from Jersey milk homogenized at 2,000 p.s.i. Amount of propionic culture added to 200 lb. of milk: *(1)* none; *(2)* 5 ml., and *(3)* 20 ml.

inoculum was criticized for bloat and irregular eyes, whereas cheese in Trial 1 was not.

The data (Table 3) show a comparison of nonhomogenized and homogenized milk cheese salted in brine without and with added KCl. The average score for six comparisons of nonhomogenized milk cheese was in favor of brine containing added KCl. Less bitterness resulted when KCl was added, but without it the cheese was judged unclean in flavor. Cheese salted in brine with added KCl

TABLE 2

The influence of additives to milk upon the quality of 3-mo.-old Swiss-type cheese

Types of additive	Trial	Score per item studied			
		Flavor	Holes and appearance	Texture	Total [e]
None (control)	1[a]	27	20	17	79
	2[b]	26	17	13	71
KCl and $MnCl_2 \cdot 2H_2O$[c] (I)	1	26	21	18	80
	2	26	16	12	69
Lactobacillus casei[d] (II)	1	28	22	19	84
	2	30	21	17	83
(I) and (II)	1	28	21	18	82
	2	28	19	15	77

[a] Propionic culture added at the rate of 20 ml/200 lb. milk.
[b] Propionic culture added at the rate of 40 ml/200 lb. milk.
[c] 40 g. KCl + 3 mg. $MnCl_2 \cdot 2H_2O$/200 lb. milk.
[d] 40 ml. peptonized milk broth culture/200 lb. milk.
[e] No criticism on salt and finish.

TABLE 3

The influence of added KCl to brine upon the quality of 3-mo.-old Swiss-type cheese[a] (Average of six lots each)

Treatment of milk	KCl added[b]	Average score per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
Homogenization at 0 p.s.i.	None	27.3	16.5	15.2	74.0
	Added	27.8	18.2	16.2	77.2
Homogenization at 2,000 p.s.i.	None	27.7	16.5	15.0	74.2
	Added	28.3	16.5	14.5	74.3

[a] All cheese made with 40 ml. propionic culture/200 lb. milk.
[b] Added to brine at the rate of 200 mg/liter.
[c] No criticism on salt and finish.

showed slightly superior holes, appearance, and texture. A similar comparison with homogenized milk cheese failed to show any significant difference in the quality of cheese attributable to added KCl.

DISCUSSION

From the data obtained in this study, it is evident that the amount of inoculum of propionic culture plays an important part in the quality of the ripened cheese. It seems advantageous to adjust the inoculum to the treatment received by the milk.

The observed beneficial effect of added *L. casei* in the manufacture of Swiss-type cheese confirms the results obtained with Swiss cheese *(2)*. Failure of K^+ and Mn^{++} to improve the quality of the ripened cheese may be difficult to explain, unless one considers the observations made by Kiuru *(6)* that KCl retards propionic fermentation. Thus, it seems that whereas added KCl is beneficial to lactobacilli in the ripening of Cheddar cheese *(5)*, its presence in Swiss-type cheese is undesirable because of its inhibitory effect upon propionic fermentation.

ACKNOWLEDGMENT

The authors are indebted to F. E. Potter for assisting in the judging of the cheese.

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FREEZING BOVINE SEMEN. III. EFFECT OF FREEZING RATE ON BOVINE SPERMATOZOA FROZEN AND STORED AT —79° C.[1,2]

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SUMMARY

These experiments show the effect of various freezing rates on the decreases in the percentages of motile spermatozoa during freezing and storage at —79° C. Spermatozoa diluted in heated fresh skimmilk showed a progressive decline in motility, irrespective of freezing rate when ampules were removed at —10, —15, —20, —35, —50, and —79° C. Greatest losses in motility with 12 freezing rates occurred between —10 and —35° C. Samples thawed immediately after reaching —79° C. showed the highest motility when a rate of 10° C. per minute from —15 to —50° C. was used. However, following storage for ten days at —79° C. there was a smaller loss of motile spermatozoa with a rate of 5° C. per minute. Very rapid freezing (70° C. per minute) was extremely detrimental; 75% of the total motile spermatozoa present before freezing were lost during freezing and storage for ten days, as compared to only 40% with a freezing rate of 10° C. per minute from —15° C. Similar, but less pronounced, harmful effects were observed when semen diluted in whole milk and yolk-citrate was frozen rapidly. In a storage trial for 0, 2, 4, 8, 16, and 32 wk., the optimum freezing rate for semen diluted in fresh skimmilk or homogenized milk was 1° C. per minute from +5 to —15° C., 5° C. per minute from —15 to —50° C., and rapidly (approximately 20° C. per minute) from —50 to —79° C. The optimum rate for semen diluted in 1:4 egg yolk–citrate was 3° C. per minute from +5 to —15° C. and 5 or 10° C. per minute from —15 to —50° C. The greatest decline in spermatozoan survival occurred during the first 4 wk., regardless of diluents or freezing rates.

British scientists demonstrated that the use of glycerol and slow cooling permitted successful freezing and storage of bull semen at —79°C. *(4, 7, 10, 11)*. Using an egg yolk–citrate diluent, Polge *(8)* found that the greatest loss in motility of spermatozoa during freezing occurred at temperatures between —15 and —20° C. However, less damage occurred if the spermatozoa were cooled quickly, over a period of about 2 min., between —15 and —25° C. VanDemark *(12)*, also using a yolk-citrate diluent, reported that cooling spermatozoa slowly to —79° C. at a rate of 0.25 or 0.5° C. per minute was inferior to rates of 1 to 4° C. per minute. He also observed *(13)* that cooling at a rate of 2° C. per minute from +5 to —19° C. and 4° C. per minute from —19 to —79° C. resulted in higher spermatozoan survival than cooling at a rate one-fourth as fast, or four times as fast.

Received for publication August 21, 1957.

[1] Authorized for publication on August 14, 1957, as Paper No. 2186 in the journal series of the Pennsylvania Agricultural Experiment Station.

[2] Supported in part by a grant from the Pennsylvania State Association of Artificial Breeding Cooperatives.

[3] A portion of the data contained in this paper is from a thesis submitted by one of the junior authors (L.A.M.) to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Master of Science.

The literature contains only limited information on the freezing rate required to obtain maximum recovery of bull spermatozoa after freezing and thawing in milk diluents. Erickson *et al. (2)* studied various freezing rates in both egg yolk–citrate and heated homogenized milk diluents, and found that over the crucial range of from —15 to —20° C., 5° C. per minute permitted a higher recovery of motile spermatozoa in yolk-citrate; whereas, a slower rate of 2° C. per minute was better with the milk diluent. Graham and Marion *(3)* also observed that slower freezing rates were required when freezing bull spermatozoa in a whole milk diluent rather than in yolk-citrate. The best rates of motility were obtained when yolk-citrate diluted semen was frozen between 3 and 5° C. per minute and when whole milk diluted semen was frozen between 2 and 3° C. per minute.

This paper reports the results of a series of experiments designed to determine more specifically the optimum rates of freezing bull spermatozoa over the range of from +5 to —79° C. in various diluents, with primary emphasis on heated skimmilk.

EXPERIMENTAL PROCEDURE

Freshly collected semen was diluted so that the initial concentration of spermatozoa was twice the final concentration of 15×10^6 motile spermatozoa per milliliter of diluted semen. Immediately after initial dilution, the samples were cooled to 5° C. over a 3- to 4-hr. period and an equal volume of diluent containing glycerol was added in five equal portions at 6-min. intervals. The final concentrations of glycerol by volume were as follows: heated fresh skimmilk, 13%; heated homogenized milk, 10%; and egg yolk–citrate, 7%. The skimmilk used in these experiments was fortified with condensed skimmilk. In yolk-citrate, the glycerol replaced an equal volume of citrate buffer, and the egg yolk was held constant at 20% by volume. In Experiments 1 and 3, the glycerol fraction of diluter also contained 2.5% fructose (w/v), to provide a final concentration of 1.25% fructose. The final diluted and glycerolated semen contained 1,000 units each of penicillin and streptomycin per milliliter.

Glycerol equilibration time varied with the design of each experiment. In Experiment 1, the equilibration period ranged from 2 to 12 hr.; whereas, in Experiments 2 and 3, a 6-hr. equilibration period was used.

All samples were frozen in 1-gal. Dewar flasks containing one liter of 95% ethyl alcohol. The temperature of the baths was lowered by the manual addition of crushed dry ice. Powdered dry ice was used when more rapid decreases in temperature of the bath were necessary.

The percentages of motile spermatozoa were estimated immediately after the frozen samples were thawed in a water bath at 5° C. To reduce bias, all treatments were coded and the motilities were estimated by a trained assistant who had no knowledge of the particular treatment used with the sample being examined.

In all trials, a Minneapolis Honeywell Automatic Temperature Recorder (Type 153) was used. In Experiment 2, the odd-numbered leads were con-

nected as Thermocouple A and the even numbers as Thermocouple B. Thermocouple A recorded the temperature of the bath, and Thermocouple B was placed in a 10 × 75 mm. test tube containing 1 ml. of diluted semen. With this arrangement, a reading was recorded on a scroll of graph-paper every 30 sec., for the bath and for the semen.

In Experiments 1 and 3, a portable Brown Potentiometer calibrated to read in degrees Centigrade also was used. The M.H. Automatic Temperature Recorder instrument was calibrated against the portable instrument. By connecting all points of the M.H. instrument to one thermocouple, the temperature within the tube of semen was recorded every 15 sec. The portable instrument was used for obtaining the bath temperature.

RESULTS

Experiment 1. It is common practice to freeze semen by gradual cooling to —15° C., followed by more rapid lowering of the temperature after —15° C. This experiment was designed to test the effect of various freezing rates before and after —15° C., upon the motility of spermatozoa during the freezing process and following storage for ten days at —79° C. in heated skimmilk diluent. Ampules were withdrawn for motility estimation from the freezing bath at the following within-ampule temperatures: —10, —15, —20, —35, —50, and —79° C. All semen samples had transformed to the crystalline state before removal, except those withdrawn at —10° C. From +5 to —15° C., freezing

TABLE 1

Effect of various freezing rates before and after —15° C., on the motility of spermatozoa frozen in heated skimmilk diluent

(Mean per cent motility of 13 ejaculates)

Freezing rate per min. from —15 to —50° C.	Freezing rate per min. from +5 to —15° C.	Motility at 5° C. prior to freezing	Motile spermatozoa in ampules removed when ampule temperature reached (°C.) —10	—15	—20	—35	—50	—79
		(%)						
5° C.	1° C.	59	54	51	49	43	43	39
	3° C.	59	56	51	47	44	41	37
	5° C.	59	54	52	49	45	39	36
	Av.	59	55	51	48	44	41	37
10° C.	1° C.	59	54	50	50	45	43	41
	3° C.	59	55	53	52	48	44	42
	5° C.	59	54	51	49	42	42	39
	Av.	59	54	51	50	45	43	41
20° C.	1° C.	59	55	52	51	47	44	37
	3° C.	59	54	54	50	47	44	41
	5° C.	59	52	52	49	46	40	36
	Av.	59	54	53	50	47	43	38
Rapid[a]	1° C.	59	52	51	46	44	42	30
	3° C.	59	54	51	49	47	42	34
	5° C.	59	53	53	48	45	41	30
	Av.	59	53	52	48	45	42	31

[a] Approximately 70° C. per minute.

rates of 1, 3, and 5° C. per minute were used; whereas, from —15 to —50° C. the semen was frozen at rates of 5, 10, and 20° C. per minute, and rapidly. Rapid freezing was obtained by adding finely powdered dry ice to the alcohol bath, and resulted in a freezing rate of approximately 70° C. per minute from —15 to —70° C. With the 5, 10, and 20° C. per minute rates, the temperature of the bath was permitted to drop as rapidly as possible from —50 to —79° C. by the addition of crushed dry ice (approximately 20° C. per minute).

The mean percentages of motile spermatozoa are presented (Table 1). A highly significant interaction was observed ($P<0.01$) between the freezing rates from —15 to —50° C. and the temperatures at which ampules were removed for motility estimations. This interaction resulted primarily from the effect of rapid freezing (70° C. per minute) from —15 to —50° C. on the motility of spermatozoa in ampules withdrawn from the freezing bath at —79° C. Therefore, it appears that the detrimental effect of extremely rapid freezing from —15° C. on spermatozoan motility was not fully exerted until the temperature of the semen was below —50° C.

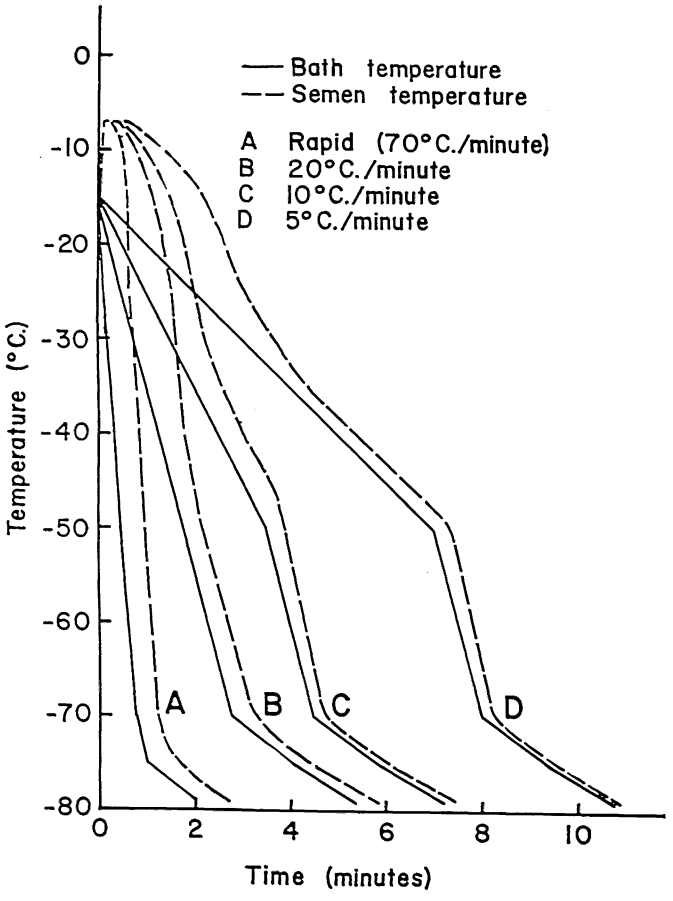


FIG. 1. Effect of lowering freezing bath temperature at four different rates between —15 and —50° C., upon the within-ampule cooling rate of semen diluted in skimmilk.

As shown (Table 1), regardless of freezing rate there was a progressive decrease in spermatozoan motility as the temperature of the semen was lowered. The differences in spermatozoan motility observed in ampules withdrawn from the bath at the various temperatures were highly significant ($P<0.01$). In all cases, a greater decrease in motility occurred between the ampule temperatures of —10 and —20° C. than with any other 10° segment of the freezing curve. The corresponding bath temperatures were —12 and —33° C., using the optimum freezing rate of 3° C. per minute from +5 to —15° C. and 10° C. per minute from —15 to —50° C. The relationship between bath and ampule temperatures for all freezing rates from —15 to —50° C. is shown (Figure 1). With the freezing rate of 1° C. per minute from +5 to —15° C., the temperature of the freezing bath was only 0.5° C. lower than that of the semen within the ampule; whereas, with rates of from 3 to 5° C. per minute, the temperature of the bath was 2° lower than that of the semen. As shown (Figure 2), with the best freezing rate (3° C. per minute from +5 to —15° C. and 10° C. per minute from —15 to —50° C.), 28% of the motile spermatozoa present before

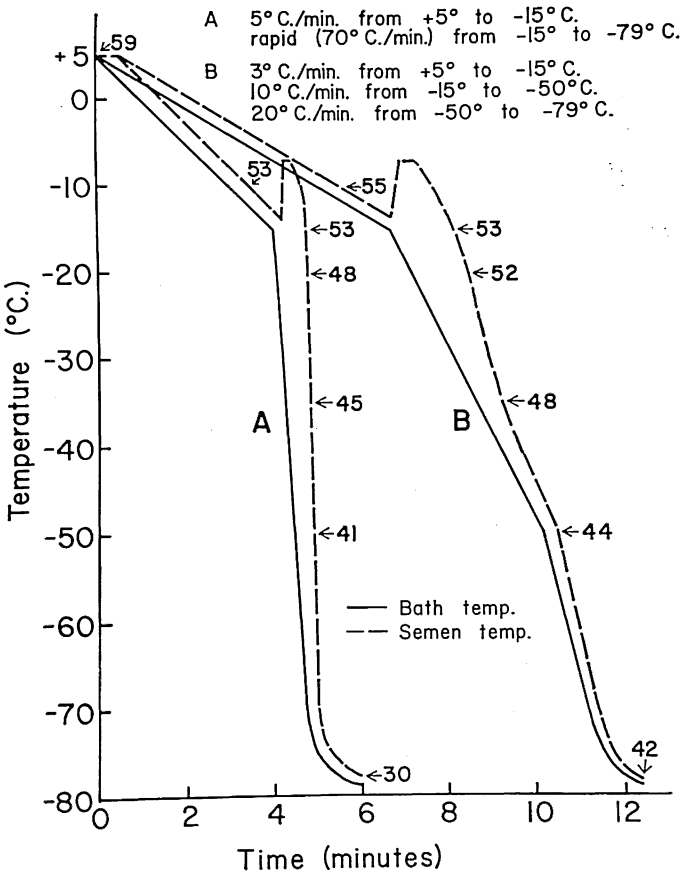


FIG. 2. The decline in the per cent of motile spermatozoa in ampules of semen withdrawn at various temperatures during freezing at two rates.

freezing failed to survive the freezing process. With the poorest freezing rate (5° C. per minute from +5 to —15° C. and rapid from —15 to —50° C.), 49% of the sperm failed to survive the freezing process. Although the highest percentages of motile spermatozoa surviving the freezing process were obtained with a freezing rate of 3° C. per minute from +5 to —15° C. and 10° C. per minute from —15 to —50° C., the differences among the three freezing rates from +5 to —15° C. were not statistically significant.

A separate analyis of variance was conducted on the effect of the 12 freezing rates on the percentages of motile spermatozoa observed immediately after freezing, and those observed in duplicate samples stored for ten days at —79° C. The mean motilities are presented (Table 2). A highly significant interaction

TABLE 2

Effect of various freezing rates on the motility of spermatozoa in heated skimmilk after storage at —79° C. for zero and ten days

(Mean per cent motility of 13 ejaculates)

Freezing rate per min. from —15 to —50° C.[a]	Freezing rate per min. from +5 to —15° C.								Percentage units of difference
	1° C.		3° C.		5° C.		Average		
	(Storage at —79° C.)								
	0 day	*10 days*	*0 day*	*10 days*	*0 day*	*10 days*	*0 day*	*10 days*	
5° C.	39	38	37	36	36	33	37	36	1.0
10° C.	41	35	42	36	39	33	41	35	6.0
20° C.	37	33	41	32	36	28	38	31	7.0
Rapid	30	15	34	16	30	14	31	15	16.0

[a] 20° C. per minute from —50 to —79° C., except for rapid freezing rate, which approximated 70° C. per minute.

between freezing rates from —15 to —50° C. and storage intervals was observed. This is explained by the fact that the slower the freezing rate from —15 to —50° C., the higher were the percentages of motile spermatozoa surviving storage for ten days at —79° C. Although a freezing rate of 10° C. per minute from —15 to —50° C. resulted in a higher percentage of motile spermatozoa immediately after freezing, the slower rate of 5° C. per minute gave slightly higher survival of spermatozoa after storage at —79° C. for ten days. With the extremely rapid rate of 70° C. per minute from —15 to —70° C., an additional 52% of the sperm surviving initial freezing were killed after ten days of storage at —79° C. In this experiment, the rapid rate of freezing from —15° C., and subsequent storage for ten days at —79° C., resulted in a loss of 75% of the motile spermatozoa present before freezing.

Based on the least significant difference between means, a significantly ($P<0.05$) higher percentage of motile spermatozoa after freezing and storage at —79° C. for ten days was obtained with the freezing rate of 3° C. per minute from +5 to —15° C. than at 5° C. per minute. The differences between 1 and 3° C. per minute and 1 and 5° C. per minute were not significant.

Experiment 2. After observing the detrimental effect of rapid freezing from —15 to —79° C., on the motility of spermatozoa diluted in heated skim-

milk, a similar experiment was designed to include heated homogenized milk and 1:4 egg yolk–citrate diluents, as well as heated fresh skimmilk. A uniform freezing rate of 3° C. per minute was used from +5 to —15° C. Freezing rates of 10° C. per minute, 20° C. per minute, and rapid (approximately 70° C. per minute) from —15° C. were employed. The rates of 10 and 20° C. per minute were controlled to —50° C., after which the samples were frozen as rapidly as possible to —79° C. (approximately 20° C. per minute) by the addition of crushed dry ice. Each of ten ejaculates was diluted in each of the three diluents. The mean percentages of motile spermatozoa in the samples thawed immediately after freezing are presented (Table 3).

TABLE 3

Comparison of the motilities of spermatozoa in milk and yolk-citrate diluents when frozen to —79°C. at various rates and thawed immediately

(Mean per cent motility of ten ejaculates)

Freezing rate in °C. per min.	Heated skimmilk	Heated homogenized milk	1:4 Egg yolk–citrate	Average
10° C.[a]	45	50	51	49
20° C.[a]	40	48	50	46
Rapid[b]	28	33	42	34
Av.	38	44	48	

[a] Between —15 and —50° C.; 20° C. per minute after —50° C.
[b] Approximately 70° C. per minute.

Analysis of variance showed a highly significant difference due to freezing rates ($P<0.01$) and a significant difference among diluters ($P<0.05$). Using the least significant difference between means to test differences between diluters, both egg yolk–citrate and heated homogenized milk were significantly ($P<0.05$) better than heated skimmilk.

The interaction between freezing rates and diluters was highly significant ($P<0.01$). In general, ejaculates frozen rapidly in heated milk diluents were lower in motility than portions of the same ejaculates frozen rapidly in yolk-citrate; and samples frozen in homogenized milk were superior to those frozen in skimmilk. However, the rapid freezing rate was detrimental to spermatozoan survival, irrespective of the diluter used.

As shown (Table 3), the mean differences among the motilities of samples frozen at the rates of 10 and 20° C. per minute were small, regardless of the diluter used. However, in all cases the mean motility of samples cooled at 10° C. per minute was higher than of those cooled at 20° C. per minute.

Experiment 3. This experiment was designed to investigate the effect of various freezing rates on the survival of spermatozoa after storage at —79° C. for 32 wk. Each of 16 ejaculates was frozen in heated skimmilk, heated homogenized milk, and 1:4 egg yolk–citrate diluents at the following rates: 1 and 3° C. per minute from +5 to —15° C.; 5 and 10° C. per minute from —15 to —50° C., and approximately 20° C. per minute from —50 to —79° C. Sufficient ampules of diluted semen were prepared to permit storage at —79° C.

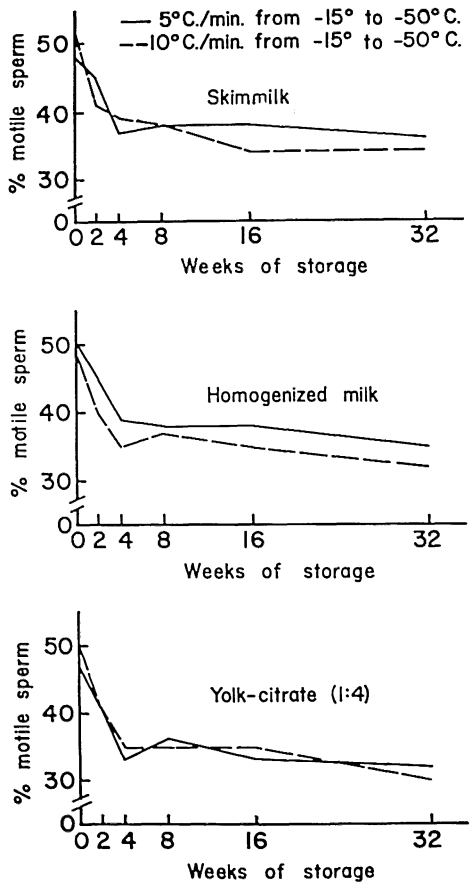


FIG. 3. Effect of two freezing rates from —15 to —50° C., on spermatozoan survival during storage at —79° C. for 32 wk. in heated milk and yolk-citrate diluents.

for intervals of 0, 2, 4, 8, 16, and 32 wk. The final diluted semen contained 1.25% fructose (w/v).

An analysis of variance involving 1,152 motility estimations revealed no significant differences among freezing rates or among diluters. However, a highly significant difference existed among storage intervals. The changes in motility after storage for varying intervals up to 32 wk. are shown (Figure 3). Irrespective of diluents and freezing rates, the major portion of the decrease in spermatozoan motility occurred during the first 4 wk. of storage.

The interaction between freezing rates from +5° to —15° C. and diluents was highly significant; whereas, that between freezing rates from —15 to —50° C. and diluents was significant at the 5% level of probability. Table 4 shows that spermatozoa cooled at 1° C. per minute from +5 to —15° C., and 5° C. per minute from —15 to —50° C., in milk diluents, gave slightly higher survival of spermatozoa after 32 wk. of storage than that observed in samples frozen at the other rates. With yolk-citrate, the faster rate of 3° C. per

TABLE 4

Effect of freezing rates and diluents on the motility of spermatozoa after 32 wk. of storage at —79° C.

(Average of 16 ejaculates and six storage intervals)

Diluent	Freezing rate per minute from +5 to —15° C.	Freezing rate per minute from —15 to —50° C.[a]		Average
		5° C.	10° C.	
Heated skimmilk	1° C.	42	40	41.0
	3° C.	39	39	39.0
	Av.	40.5	39.5	40.0
Heated homogenized milk	1° C.	42	38	40.0
	3° C.	40	38	39.0
	Av.	41.0	38.0	39.5
Egg yolk–citrate (1:4)	1° C.	36	37	36.5
	3° C.	38	39	38.5
	Av.	37.0	38.0	37.5

[a] All frozen 20° C. per minute after —50° C.

minute from +5 to —15° C. and 5 or 10° C. per minute from —15 to —50° C. gave a somewhat higher survival of spermatozoa after 32 wk. of storage at —79° C. Although the differences among diluents were not significant, a slightly higher survival of spermatozoa was obtained in milk diluents than in yolk-citrate diluent, after 32 wk. of storage at —79° C.

The interactions of ejaculates × freezing rates from —15 to —50° C., and ejaculates × diluents were highly significant ($P<0.01$); whereas, those of ejaculates × freezing rates from +5 to —15° C. were significant at the 5% level of probability.

A given set of conditions for freezing bull spermatozoa did not produce optimum recovery of spermatozoa for all bulls, or even for different ejaculates from the same bull. This was shown by the highly significant third-order interaction involving ejaculates, diluents, freezing rates from +5 to —15° C., and freezing rates from —15 to —50° C. In spite of the significant inconsistency from one ejaculate to another, the over-all patterns of interaction (shown Table 4) were significant.

DISCUSSION

These studies show that spermatozoa frozen to —79° C. in heated milk diluents are less resistant to rapid decreases in temperature (70° C. per minute) than spermatozoa frozen in egg yolk–citrate diluent. However, slower freezing rates after —15° C. (5 or 10° C. per minute) gave higher spermatozoan survival in both milk and yolk-citrate diluents. The detrimental effect of rapid freezing after —15° C. on spermatozoan motility in skimmilk diluent was exerted not only during the freezing process but also during subsequent storage at —79° C. for ten days. Of the motile spermatozoa present before freezing, 75% were destroyed after rapid freezing and storage at —79° C. for ten days, as compared to only 40% of those frozen at 10° C. per minute from —15 to —50° C. and stored for ten days.

The results of these experiments clearly show that the term rapid freezing, from —15 to —79° C., as used by various workers, is too general. The actual rate of temperature decline commonly referred to as rapid may vary sufficiently to account for some of the differences in freezability results reported by various workers.

Buch *et al. (1)*, using a yolk-citrate diluent, reported that the greatest decline in per cent of motile spermatozoa occurred during the first 14 days of storage at —79° C. In Experiment 3, the decline extended beyond 14 days and there was a considerable loss in motile spermatozoa between 2 and 4 wk. of storage. The decline between 4 and 32 wk. of storage was very small in both milk and yolk-citrate diluents. Spermatozoa in heated milk diluents retained a slightly higher percentage of motile spermatozoa after 32 wk. of storage at —79° C. than those stored in yolk-citrate diluent.

The results in Experiment 3 showed that a given set of conditions for freezing and storing bull spermatozoa did not give optimum recovery of motile spermatozoa for all bulls, or even for different ejaculates from the same bull. This has been observed by other workers *(1, 6, 9)*.

Based upon the motility of spermatozoa in ampules of semen removed at various temperatures during the freezing process, Experiment 1 showed a progressive decline in motility, with the major portion of the decrease occurring between bath temperatures of approximately —10 and —35° C. This was true of all freezing rates except the 70° C. per minute rate, where a large decrease also occurred between —50 and —79° C. These results agree quite closely with the previous work of Marsh *(5)*, Polge *(8)*, and Lovelock and Polge *(4)*. When spermatozoa were examined immediately after freezing, it was observed that passing through the critical temperature range at 10 or 20° C. per minute, rather than at 5° C. per minute, reduced the loss of motile spermatozoa during the freezing process. However, when samples were examined after ten days of storage at —79° C., it was observed that the faster freezing rates were more detrimental to spermatozoan survival than the slower rate of 5° C. per minute through the critical temperature range.

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INFLUENCE OF SEMINAL PLASMA AND MATURITY OF BOVINE SPERMATOZOA UPON THEIR FREEZABILITY

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SUMMARY

Two experiments were conducted to determine whether the greater postfreezing revival of spermatozoa from the second over the first ejaculate is attributable to differences in seminal plasma or to differences in the cells. In both experiments, spermatozoa were frozen in yolk-citrate diluter. In the first experiment, seminal plasma was interchanged between first and second ejaculates. The superiority of spermatozoa from the second over the first ejaculates remained, whether plasma from the same or the other ejaculate was in the medium or whether the plasma was removed. In the second experiment, the freezability of spermatozoa in the cauda epididymis was superior to that in the ampulla, although not significantly so. The evidence from these two experiments indicates that the superiority in freezability of spermatozoa from the second over the first ejaculate can be attributed to characteristics of the cells and not to differences in seminal plasma.

The preservation of bull spermatozoa by freezing has been successful in many laboratories and artificial breeding organizations and is practiced routinely in some bull studs. About one-third to one-half of spermatozoa are killed during freezing and thawing. Prevention of this wastage may depend upon greater knowledge of the factors associated with survival and death during the freezing process. Available data have suggested that maturity of spermatozoa or changes in seminal plasma might be some of these factors. Two sets of observations from this laboratory *(6)* and a report by Kinney and VanDemark *(11)* indicate that a greater percentage of spermatozoa survives freezing in the second than in the first ejaculate, when they are collected a few minutes apart. Kinney and VanDemark also reported increased survival in successive ejaculates through the fourth, and decreases thereafter. They also observed less resistance to freezing with spermatozoa from the epididymis than with spermatozoa from the more exterior parts of the tract. Elliott *et al. (7)* observed greater freezability of spermatozoa collected after a lapse of from three to seven days from the previous collection than after a lapse of only one day. Progressive improvement in freezability of spermatozoa through four successive ejaculates *(6)* was also noted.

There is some evidence that the chemical composition of spermatozoa in first and second ejaculates differ. Lindahl and Kihlström *(12)* reported a decrease in specific gravity of spermatozoa with successive ejaculates. They interpreted this to mean that dehydration of spermatozoa takes place with increase in maturity. This change in specific gravity has been confirmed by Ohms and Willett *(14)*.

Received for publication July 3, 1957.

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Furthermore, the composition of seminal plasma changes with successive ejaculates. Changes which have been observed include freezing-point depression *(15)*, pH *(1, 5, 7)*, and phosphomonoesterases *(9)*. Branton *et al. (3)* have shown that sexual excitement can affect chemical composition of seminal plasma, probably by changing the amounts of secretions from the different accessory glands.

These observations raise the question as to whether these or other changes in spermatozoa or plasma or in both are related in some way to freezability. To aid in answering this question, two experiments were carried out and are reported in this paper.

EXPERIMENT I

This experiment was designed to determine if a difference in plasma is responsible for the greater freezability of spermatozoa in the second ejaculate than in the first. Seminal plasma was interchanged between ejaculates or removed from the medium.

Experimental procedure. First and second ejaculates from bulls were collected a few minutes apart. Semen from each ejaculate was placed in five round-bottom test tubes, each containing 5 ml. of yolk-citrate, to give a concentration of 30×10^6 spermatozoa per milliliter. The yolk-citrate diluter consisted of one part yolk and three parts of 2.9% sodium citrate dihydrate and was clarified by centrifuging at 2,500 r.p.m. (about 800 g.) at room temperature for 30 min. This treatment does not reduce the ability of this diluter to maintain livability of spermatozoa during storage at 5° C. *(16)*. Diluted semen in each of the five tubes from each ejaculate received one of the following treatments: *(a)* Uncentrifuged; *(b)* centrifuged twice at 3,100 r.p.m. (2,200 g.) for 3 min. and each time resuspended in supernatant; *(c)* centrifuged as above, removed supernatant, resuspended in yolk-citrate, centrifuged again, removed supernatant, and resuspended in 4.5 ml. yolk-citrate and 0.5 ml. plasma from same ejaculate; *(d)* same as *c*, except that plasma from other ejaculate from the same bull was added after second washing; *(e)* same as *c*, except that 5 ml. of yolk-citrate only was added after second washing.

The seminal plasma added to treatments *c* and *d* had been prepared by centrifuging whole semen at 20,000 r.p.m. (25,000 g.) for 25 min. in a refrigerated Spinco preparative centrifuge, and pipetting off the clear supernatant.

After these treatments, the diluted semen was cooled. Five or 6 hr. after collection, glycerolation was carried out by adding, in five steps, an equal volume of yolk-citrate containing glycerol, at a level of 14% by volume. Then six 1-ml. portions from each test tube were placed in separate, smaller tubes. On the forenoon of the following day, the semen was frozen in an alcohol bath with solid carbon dioxide by cooling from 5 to —12° C. in approximately 14 min., then cooling rapidly with pulverized solid carbon dioxide. Shortly after freezing, the samples were thawed in a water bath at 5° C. with gentle agitation. Motility estimations were then made on the frozen and nonfrozen samples by two people independently and without knowledge of the treatments of the indi-

vidual samples. Estimations were made of spermatozoa from two tubes selected at random from each treatment and were averaged.

Semen from ten collections from seven bulls was employed in this experiment. The volumes of the first and second ejaculates averaged 5.4 and 6.9 ml., and number of spermatozoa per milliliter, 1,785 and 1,529 $\times 10^6$, respectively. Little effort was made to excite the bulls prior to the first ejaculate.

Results. Results are summarized (Table 1). Differences among treatment means within ejaculates are not significant, but the average motility for second

TABLE 1

Progressive motility of spermatozoa from first and second ejaculates, when the spermatozoa were washed, the plasma interchanged or replaced with yolk-citrate, and frozen
(Figures are averages of ten collections)

	Treatment				
	A	B	C	D	E
			Washed		
	Uncentrifuged	Centrifuged, remixed	Same plasma added	Other plasma added	Diluter added
	(%)				
1ST EJACULATE					
Unfrozen	52	51	52	52	50
Frozen	28	27	25	27	24
Revival	54	53	48	52	48
2ND EJACULATE					
Unfrozen	62	63	63	63	61
Frozen	46	41	42	44	44
Revival	74	65	67	70	72

ejaculates is highly significantly greater than for first ejaculates ($P<0.001$). This statement applies whether the diluted semen was or was not frozen. When post-thaw motility means for ejaculates were adjusted by covariance to the same nonfrozen motilities, the difference between ejaculates was still highly significant.

EXPERIMENT II

This study was carried out to determine if spermatozoa from different parts of the bull's reproductive tract differ in their ability to survive freezing.

Experimental procedure. Reproductive organs were dissected from 15 slaughterhouse bulls about 15 min. after they were killed. About 1 hr. later, spermatozoa were flushed from the ampullae, small tubes of the ducti deferentia, and cauda epididymides with yolk-citrate, which consisted of 5% by volume of a 1 *M* solution of sodium citrate, 25% yolk, and 70% distilled water. Spermatozoa were obtained from the caput epididymides by incising the organ in several places and squeezing the spermatozoa into yolk-citrate. Spermatozoa from the different areas were brought to roughly the same concentration in the diluter as judged under the microscope, and were then placed in a refrigerator.

A subsample was removed from each tube and stained with eosin and nigrosin *(2)* on a slide, for later counting of live and dead spermatozoa. Numbers of

proximal and distal protoplasmic droplets were also determined. Approximately 200 spermatozoa were counted in each preparation. Counts were not made of frozen and thawed spermatozoa because of interference by glycerol *(13)*.

The freezing procedure was as follows: About 2.5 hr. after removal of spermatozoa from the organs, the diluter containing the spermatozoa was glycerolated in five steps over a ½-hr. period with the following diluent: 8.6% by volume of a 1 *M* solution of sodium citrate, 25% yolk, 52.4% distilled water, and 14% glycerol. The diluted semen was then frozen as in Experiment I. About 2 hr. later, the samples were thawed and examined.

Results. The data are summarized (Table 2). Motility of nonfrozen spermatozoa from cauda epididymides, small tubes of the ducti deferentia, and ampullae was practically the same. After freezing, however, both progressive and total

TABLE 2

Average motile and live spermatozoa from different parts of the reproductive tract of the bull when frozen and not frozen
(Figures are averages of 15 bulls)

	Epididymides		Small ducts	Ampullae
	Caput	Cauda		
	(%)			
TOTAL MOTILITY				
Unfrozen	2	70	71	70
Frozen	1	41	32	37
Revival	–	59	45	53
PROGRESSIVE MOTILITY				
Unfrozen	0	59	60	60
Frozen	0	29	20	27
Revival	0	49	33	45
LIVE SPERM				
Unfrozen	88	90	85	83

motility of spermatozoa from the small ducts were significantly lower ($P < 0.05$) than those from the cauda epididymides or ampullae. The differences between the latter two were not significant. Practically no spermatozoa from the caput were motile, although they were alive, as indicated by the eosin-nigrosin stain. There were no significant differences in percentages of live spermatozoa among the four locations.

DISCUSSION AND CONCLUSION

In Experiment I, motilities of spermatozoa were similar whether they were in the presence of seminal plasma from the same or the other ejaculate, or in virtual absence of plasma during the freezing process. These results indicate, *(a)* that differences in plasma are not responsible for the superiority in freezability of spermatozoa in the second over those in the first ejaculate, when frozen and thawed, and *(b)* that these differences in ability to survive freezing appear to be characteristic of the cells.

In Experiment II, the spermatozoa from the small tubes of the ducti deferentia had lower motility after freezing and thawing than those from the cauda

epididymides or from the ampullae. Nevertheless, because of the very small number of spermatozoa that can be recovered from the small tubes of the ductus deferens, their reduced motility would not greatly influence the results observed in successive ejaculates. The trend (although not significant) of increased percentages of motile or live spermatozoa obtained at regions progressing from the ampulla to the cauda epididymis is in agreement with the observations made in freezability of spermatozoa from first and second ejaculates.

The number of protoplasmic droplets and their location on the spermatozoa from different parts of the tract, agreed very closely with the data reported by Branton and Salisbury *(4)*.

On the basis of the evidence obtained from these two experiments, it can, therefore, be concluded that the superiority in freezability of spermatozoa from the second over the first ejaculate can be attributed to a factor or factors in the cells and not in the seminal plasma. It is of interest to note that Herman and Swanson *(10)* interchanged plasma from different bulls and observed survival at 5° C. Emmens and Blackshaw *(8)* likewise interchanged seminal plasma among semen samples from different species and then froze them. These different workers concluded that survival of spermatozoa under their respective conditions was more dependent on the characteristics of the spermatozoa themselves than upon the properties of the seminal plasma.

In Experiment I, spermatozoa were exposed to seminal plasma from time of collection to removal by washing, an interval of 0.5 to 1 hr. Changes in spermatozoa related to freezability could be brought about by plasma during this interval. If such is the case, seminal plasma could indirectly be responsible for differences in freezability of spermatozoa in first and second ejaculates, rather than a factor[s] in the cells, as the data in Experiment I suggest. However, the trend in freezability of spermatozoa from different parts of the tract tends to counter this possibility.

The specific changes in spermatozoa that are apparently associated with aging and freezability need yet to be determined.

ACKNOWLEDGMENT

The authors wish to acknowledge the generosity of Oscar Mayer and Company in allowing them to procure organs from bulls.

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ESTIMATION OF SOME VARIANCE COMPONENTS OF BOVINE SEMEN CRITERIA AND THEIR USE IN THE DESIGN OF EXPERIMENTS [1]

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SUMMARY

Semen production records of 68 bulls from five dairy breeds in four studs were used to estimate the bull, ejaculate, breed, season, and interaction variance components of five criteria used to measure semen production. The means and standard deviations of individual observations for the five criteria were: ml. of semen per ejaculate, 8.0 ± 2.4; per cent motile sperm, 66 ± 9.0; billions of sperm per ml. of semen, 1.71 ± 0.62; billions of motile sperm per ejaculate, 8.9 ± 3.9, and billions of total sperm per ejaculate, 13.6 ± 6.0. Component of variance analyses showed that the bull and ejaculate components accounted for more than 90% of the total variance in all criteria except per cent motile sperm. Consequently, these two variances were taken into account in estimating the power of the test by Tang's method. This estimate showed that there was little increase in sensitivity to be gained by taking more than ten ejaculates per bull. It was calculated that to have a 75% chance of detecting a treatment difference of 50% of the mean of motile sperm per ejaculate at the 5% level of significance would require about ten bulls per treatment and five ejaculates per bull.

Although it is common knowledge that the volume, sperm concentration, and per cent of motile sperm of bovine semen ejaculates vary greatly, the authors have found no reports in the literature indicating the size of the components of variance of these criteria for unextended semen. There are in the literature three reports having to do with the estimation and use of variance components of criteria for extended semen *(3, 4, 5)*.

The practical importance of having an estimate of these parameters lies in the fact that with them one may calculate the size of an experiment (number of replications) necessary to detect a specified difference between treatments at a specified level of significance (P_I), and with a specified probability $(1 - P_{II})$ of detecting the difference.

The present report deals with the estimation and use of some of the components of variance contributing to the total variance of ejaculate volume, per cent motile sperm, sperm concentration, motile sperm per ejaculate, and total sperm per ejaculate of dairy sires, criteria having considerable genetic and economic importance in artificial breeding.

MATERIALS AND METHODS

The data available for determining the desired variance components consisted of complete records of sperm production from 68 dairy bulls in four

Received for publication September 6, 1957.

[1] The data in this paper are, in part, from a thesis submitted by the senior author to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science, February, 1957.

large bull studs during the period June 1, 1955, through May 29, 1956. These bulls had been ejaculated at intervals ranging from one to 20 days, the modal interval between ejaculates being 1 wk. The per cent of motile sperm in the fresh unextended semen was estimated after dilution in either 2.9% sodium citrate or 0.9% sodium chloride in distilled water. Sperm concentration in the ejaculates was measured with Cenco photelometers,[2] all of which had been calibrated in the authors' laboratory. These procedures for semen examination were essentially those of Willett and Salisbury *(12)*, as modified by Salisbury *et al. (9)*, Willett and Buckner *(11)*, and Bratton *et al. (2)*.

TABLE 1

Measured sources of variation and the expectations of their mean squares

Source of variation		Expectation of mean squares
Season	S	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b} + k_2\sigma^2_s$
Breed	B	$\sigma^2_w + \sigma^2_{e:abs} + k_3\sigma^2_{a:b} + k_4\sigma^2_b$
Interaction	SB	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b} + k_5\sigma^2_{sb}$
Animal (bull)	A:B	$\sigma^2_w + \sigma^2_{e:abs} + k_3\sigma^2_{a:b}$
Interaction	SA:B	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b}$
Ejaculate	E:ABS	$\sigma^2_w + \sigma^2_{e:abs}$

The extensiveness of the available data permitted selection of bulls and ejaculates within bulls, so as to avoid the excessive computational labor involved in disproportionate subclass number component of variance analysis. The bases for selecting the bulls were: *(a)* Each bull had to have at least one ejaculate of semen in each consecutive 3-wk. period during the 52 wk. of the study and *(b)*, within a stud, each bull had to have a specified minimum number of ejaculates in each of the four 13-wk. seasons of the year. When a bull had more than the specified minimum number of ejaculates for a 13-wk. season, the excess, which was to be discarded, was chosen at random.

The analyses of variance and the component of variance analyses considered the six sources of variation shown (Table 1). The season and breed sources were considered to be fixed and factorial, the animal (bull) source to be random and nested within breed, and the ejaculate source to be random and nested

[2] These were Catalog No. 29330, Central Scientific Co., Chicago, Illinois.

TABLE 2

Number of bulls per breed per stud included in the analyses

Breed	Bull stud I	II	III	IV	Total
Holstein	3	8	7	7	25
Guernsey	2	2	8	5	17
Jersey	3	4	3	0	10
Ayrshire	3	4	0	0	7
Brown Swiss	0	4	0	5	9
Total	11	22	18	17	68

within bull-breed-seasons *(7)*. The following mixed linear model was considered to be appropriate for these data:

$$X_{hijk} = \mu + s_k + b_j + sb_{jk} + a{:}b_{ij} + sa{:}b_{ijk} + e{:}abs_{hijk} + E_{hijk},$$

where X_{hijk} is the value for the hth ejaculate, from the ith animal, in the jth breed and in the kth season. In this model, μ is the mean of the population and E_{hijk} are random errors with a mean of zero and a variance of σ^2_w. All other components of the model were assumed to be uncorrelated variables with means of zero and variances of σ^2_s, σ^2_b, σ^2_{sb}, $\sigma^2_{a:b}$, $\sigma^2_{sa:b}$, and $\sigma^2_{e:abs}$.

The expectations of the mean squares and the coefficients of the components of these expectations (shown in Table 1) were determined by Henderson's procedures *(6, 7)*.

RESULTS AND DISCUSSION

The restrictions applied in selecting the bulls whose semen records were used resulted in the distribution of the bulls among breeds and studs as shown (Table 2). There were seven ejaculates per bull-breed-season subclass for Stud I, ten for Stud II, 11 for Stud III, and 13 for Stud IV, giving a total of 308, 880, 792, and 884 ejaculates per stud, respectively, and a grand total of 2,864 ejaculates.

The averages for the five semen criteria in each stud are shown (Table 3). The standard deviations and the coefficients of variation of the criteria, calculated from the total variance, are shown (Table 4). The similarity among studs of the coefficients of variation within each criterion suggests that these estimates may be reasonably representative of bull studs in general. In fact, they agree closely with estimates for the same criteria recently reported by Bishop and Hancock *(1)* in England.

The analysis of variance for each of the five criteria in each of the four bull studs resulted in the F ratios shown in Table 5. Differences among bulls were highly significant in every test. Although 13 of the 20 tests of seasonal effects

TABLE 3
Average of semen criteria for the four bull studs

Bull stud	Volume of ejaculate	Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate
	(ml.)	*(%)*		*(×10⁹)*	
I	9.0	62	1.82	9.71	15.9
II	8.5	62	1.50	7.95	12.7
III	7.9	72	1.72	9.80	13.6
IV	7.4	65	1.88	8.85	13.7
Av.	8.0	66	1.71	8.93	13.6

were statistically significant, the actual seasonal differences were considered to be too small to be of much practical value in the design of experiments. None of the 20 F tests for differences among breeds was significant, or even approached significance. Much the same situation was true for the season-by-breed interaction.

TABLE 4

Standard deviations and coefficients of variation for all criteria for each bull stud

Bull stud	Volume of ejaculate		Motile sperm		Concentration of sperm per ml.		Motile sperm per ejaculate		Total sperm per ejaculate	
	s	CV	s	CV	s	CV	s	CV	s	CV
	(ml.)		*(%)*		*($\times 10^9$)*	*(%)*	*($\times 10^9$)*	*(%)*	*($\times 10^9$)*	*(%)*
I	2.4	41	8.7	14	0.78	43	4.3	44	7.3	46
II	2.9	34	8.5	12	0.57	38	3.9	49	6.0	47
III	2.2	28	12.3	17	0.52	30	4.1	42	5.7	42
IV	2.1	28	6.3	10	0.60	32	3.4	38	5.2	38
Av.	2.4	33	9.0	13	0.62	36	3.9	43	6.0	43

Values obtained for the components of variance are shown (Table 6). Two important findings stand out in this table: First, the ejaculate ($s^2_{e:abs}$) and the bull ($s^2_{a:b}$) estimates of variances are by far the largest components in all criteria except per cent motile sperm. Secondly, many negative values occur in the estimates of the breed (s^2_b) and the season-by-breed (s^2_{sb}) variances. But since negative variances are impossible, these negative values are considered as estimates of parameters which are very close to or equal to zero. The average of the among-stud values for each component, within each criterion, shows that the estimated breed variance (s^2_b)is zero in four of the five criteria, and that the estimated bull ($s^2_{a:b}$) and ejaculate ($s^2_{e:abs}$) variances together account for more than 90% of the total variance in all criteria except per cent motile sperm. Since these two components accounted for most of the variance, they are the only ones considered in the following discussion of the application of variance components in the design of sperm output experiments.

To calculate P_{II} for a two-treatment experiment, Tang's ϕ was computed. For the data in Table 6, the following formula for ϕ was used, as shown by Henderson *(7)*.

$$\phi = \frac{d}{2\,\sigma_{\bar{x}}}$$

In this formula, d is the treatment difference to be detected and $\sigma_{\bar{x}}$ is the standard deviation of a treatment mean. For these data the standard deviation of a treatment mean is estimated from the following formula:

$$s_{\bar{x}} = \sqrt{\frac{s^2_{a:b}}{n_a} + \frac{s^2_{e:abs}}{(n_a)(n_e)}},$$

where $s^2_{a:b}$ is the bull variance, $s^2_{e:abs}$ is the ejaculate variance, n_a is the number of bulls per treatment, and n_e is the number of ejaculates per bull.

Having ϕ, one may find P_{II} from Tang's tables of ϕ shown by Tang *(10)* and by Kempthorne *(8)*. Subtracting P_{II} from one gives the power of the test,

TABLE 5

Summary of F ratios for all studs

Source of variation	Bull stud	Degrees of freedom	Volume of ejaculate	Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate	Significance level	
								5%	1%
S	I	3	3.26	0.37	4.57	9.40	13.96	3.07	4.87
	II	3	5.50	1.97	1.60	0.60	0.94	2.79	4.20
	III	3	3.66	3.55	3.38	5.28	1.62	2.82	4.26
	IV	3	3.54	35.72	9.37	0.72	3.11	2.83	4.29
B	I	3	1.28	0.59	1.03	1.61	0.71	4.35	8.45
	II	4	1.27	0.70	0.78	0.20	0.16	2.96	4.67
	III	2	0.54	0.24	0.98	1.52	1.64	3.68	6.36
	IV	2	0.26	1.95	0.65	0.99	1.08	3.74	6.51
SB	I	9	1.67	0.45	2.09	1.72	2.32	2.37	3.40
	II	12	1.35	0.72	1.11	1.17	1.14	1.95	2.56
	III	6	0.12	0.24	0.43	0.80	0.49	2.31	3.23
	IV	6	1.59	1.60	1.09	2.10	1.88	2.32	3.26
A : B	I	7	14.41	43.92	63.75	32.71	52.92	2.05	2.72
	II	17	27.13	20.99	52.94	73.03	72.84	1.64	1.99
	III	15	46.61	6.03	46.83	25.11	26.98	1.69	2.06
	IV	14	20.69	5.43	26.29	12.52	12.23	1.71	2.10
SA : B	I	21	1.44	1.38	2.81	1.31	0.88	1.60	1.93
	II	51	1.23	2.13	1.65	1.08	1.16	1.37	1.56
	III	45	1.03	6.14	1.41	1.17	0.92	1.40	1.59
	IV	42	1.37	1.61	3.26	2.25	2.22	1.41	1.62

TABLE 6

Estimates of the magnitude of the variance components for the semen criteria for all studs

Component of variance	Bull stud	Volume of ejaculate	% Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate
s^2_s	I	0.15	−0.37	0.02	1.00	2.79
	II	0.12	0.32	0.00	−0.01	−0.01
	III	0.03	7.74	0.00	0.26	0.06
	IV	0.05	5.56	0.02	−0.02	0.41
	Av.	0.09	3.31	0.01	0.31	0.82
s^2_b	I	0.19	−7.79	0.00	1.84	−3.80
	II	0.20	−1.31	−0.01	−2.14	−5.39
	III	−0.21	−1.82	0.00	0.54	1.35
	IV	−0.16	0.39	−0.01	0.00	0.07
	Av.	0.00	−2.63	0.00	0.06	−1.94
s^2_{sb}	I	0.18	−1.29	0.03	0.34	1.14
	II	0.05	−0.49	0.00	0.03	0.06
	III	−0.04	−7.43	0.00	−0.04	−0.15
	IV	0.03	0.29	0.00	0.29	0.52
	Av.	0.06	−2.23	0.01	0.16	0.39
$s^2_{a:b}$	I	1.67	50.13	0.36	7.92	35.03
	II	3.06	17.28	0.18	11.04	26.61
	III	2.52	11.18	0.13	5.57	11.39
	IV	1.15	1.87	0.10	1.92	4.23
	Av.	2.10	20.12	0.19	6.61	19.32
$s^2_{sa:b}$	I	0.22	1.75	0.04	0.31	−0.33
	II	0.11	3.89	0.01	0.05	0.24
	III	0.01	45.69	0.00	0.16	−0.13
	IV	0.09	1.03	0.04	0.83	1.83
	Av.	0.11	13.09	0.02	0.34	0.40
$s^2_{c:abs}$	I	3.49	32.69	0.16	6.99	18.89
	II	4.69	34.58	0.14	6.13	14.82
	III	2.43	97.81	0.13	10.16	19.29
	IV	3.04	29.97	0.21	8.65	19.58
	Av.	3.66	48.74	0.16	7.98	18.14
s^2_{total}	I	5.90	75.12	0.61	18.40	53.72
	II	8.23	54.28	0.32	15.10	36.32
	III	4.75	153.16	0.26	16.64	31.80
	IV	4.20	39.12	0.36	11.66	26.66
	Av.	6.02	85.26	0.39	15.46	42.69

that is, the probability of detecting a treatment difference as large as or larger than the one used in the calculation of ϕ.

The power of the analysis of variance F test $(1-P_{II})$ was estimated for some possible two-treatment experiments with varying numbers of bulls per treatment and ejaculates per bull.

Table 7 shows for each criterion in each stud in the study the probabilities $(1-P_{II}$'s) of detecting a treatment difference of 50% of the mean at the 5% significance level, when using two to ten bulls per treatment and five to 50 ejaculates per bull. From these values, it is apparent that in most instances five to ten bulls per treatment and five to ten ejaculates per bull provide relatively good chances of detecting a difference of 50% of the mean. The very high $1-P_{II}$ values for per cent motile sperm reflect the small coefficients of variation of this criterion (shown in Table 4).

Figures 1 and 2 show, for the average stud, the per cent chance of detecting, at the 5% level of significance, a treatment difference of 25 or 50% of the mean number of motile sperm per ejaculate, for varying numbers of bulls per treatment and ejaculates per bull. These figures also show the alternative combinations of numbers of bulls per treatment and ejaculates per bull that have about the same power of the test. For example, in Figure 2, the power of the test for five bulls per treatment and ten ejaculates per bull is approximately 62%, as compared to 69% for ten bulls per treatment and one ejaculate per bull.

TABLE 7

Per cent chance of detecting a treatment difference of 50% of the mean at the 5% level of significance with varying numbers of bulls per treatment and ejaculates per bull

		Number of bulls per treatment											
		2				5				10			
		No. ejac/bull/treatment											
Criteria	Bull stud	5	10	20	50	5	10	20	50	5	10	20	50
Volume of ejaculate	I	38	43	46	46	97	98	98	99	99	99	99	99
	II	24	26	28	28	84	87	89	89	99	99	99	99
	III	26	28	28	28	87	89	92	92	99	99	99	99
	IV	36	41	43	46	96	97	98	99	99	99	99	99
	Av.	28	31	33	33	89	94	94	96	99	99	99	99
Per cent motile sperm	I	59	59	62	66	99	99	99	99	99	99	99	99
	II	86	92	92	92	99	99	99	99	99	99	99	99
	III	86	95	99	99	99	99	99	99	99	99	99	99
	IV	99	99	99	99	99	99	99	99	99	99	99	99
	Av.	82	86	92	92	99	99	99	99	99	99	99	99
Concentration of sperm per ml.	I	14	14	16	16	51	58	58	58	85	85	85	85
	II	18	18	18	18	60	65	65	65	93	93	95	95
	III	24	26	26	28	84	87	89	89	99	99	99	99
	IV	31	33	36	38	92	94	96	97	99	99	99	99
	Av.	20	20	22	22	70	73	77	77	93	93	98	98
Motile sperm per ejaculate	I	16	18	18	18	60	60	65	65	92	93	93	95
	II	11	11	11	11	37	37	37	37	67	67	67	67
	III	18	22	22	22	70	77	80	80	95	97	98	99
	IV	26	31	38	41	89	94	96	97	99	99	99	99
	Av.	16	18	18	18	58	60	65	65	92	93	93	95
Total sperm per ejaculate	I	12	12	12	12	42	46	46	46	76	77	77	77
	II	11	11	12	12	37	42	42	42	67	71	71	71
	III	18	20	22	22	65	73	77	77	95	97	98	99
	IV	28	33	38	43	89	96	97	98	99	99	99	99
	Av.	14	14	16	16	51	58	58	58	85	85	85	85

To increase one's chances of detecting a treatment difference, that is, to increase the power of the test, one has four alternatives. He may *(a)* increase the level of significance, P_I, that is acceptable (e.g., use the 10% rather than the 5% level of significance), *(b)* attempt to detect a larger difference, *(c)* use more bulls or ejaculates per treatment, and/or *(d)* reduce the magnitude of the error

variance. The final choice of power of the test, level of significance, treatment difference to be detected, and number of bulls and ejaculates to be used depends upon the resources available and upon the value of the detectable difference to the experimenter.

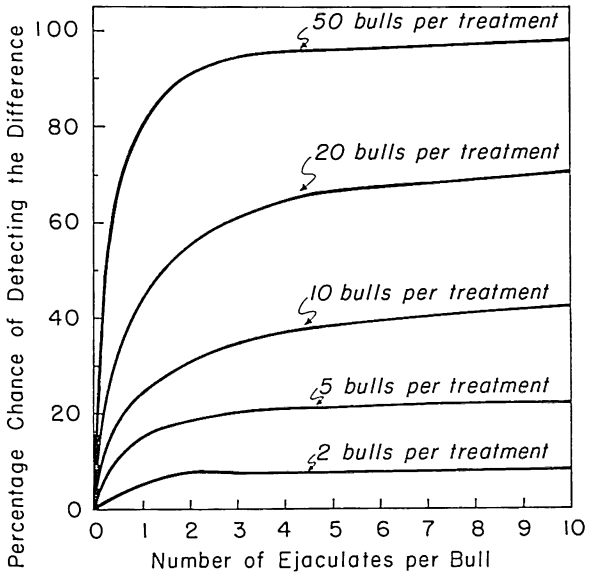


FIG. 1. Per cent chance of detecting a difference of 25% of the mean number of motile sperm per ejaculate, at the 5% significance level, for varying numbers of bulls per treatment and ejaculates per bull.

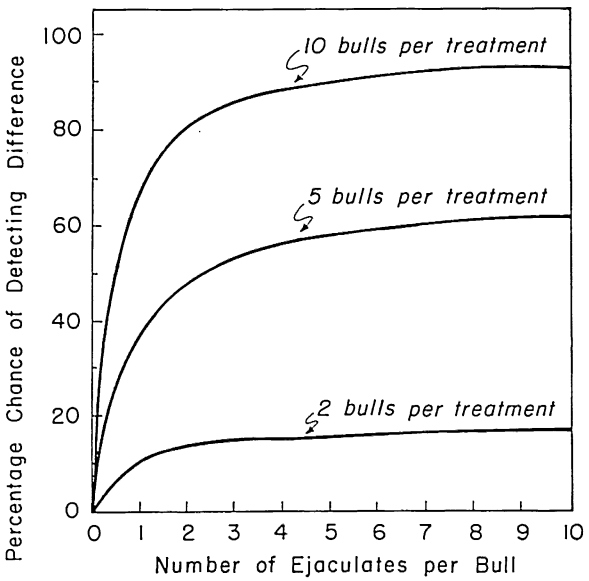


FIG. 2. Per cent chance of detecting a difference of 50% of the mean number of motile sperm per ejaculate, at the 5% significance level, for varying numbers of bulls per treatment and ejaculates per bull.

ACKNOWLEDGMENTS

The authors wish to express appreciation to the management and personnel of the New York Artificial Breeders' Cooperative, Inc., Ithaca; The Northern Ohio Breeders' Cooperative Association, Tiffin; The Eastern Iowa Artificial Breeding Association, Cedar Rapids, and the Massachusetts Selective Breeding Association, Shrewsbury, for their cooperation in obtaining these data. Thanks are also due Mrs. Sally Shipman and Mrs. Viola Perry for aid in part of the statistical analyses.

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EFFECT OF INBREEDING ON PRODUCTION IN HOLSTEINS[1]

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SUMMARY

Data collected over a 25-yr. period from the Iowa State College Holstein herd were used to study the effect of inbreeding on production. The inbreeding ranged from zero to 34% and averaged 7.4%. Records used were standardized to a 305-day-2×-M.E. base. The number of cows used in each analysis varied but, at the most, 534 were available. Intra-sire regressions, using the average of each cow's records, were -1.74 ± 0.57 lb. of butterfat, -54 ± 17 lb. of milk, and $+ 0.003 \pm 0.003\%$ of butterfat for each increase of 1% in inbreeding. There was no evidence of curvilinearity in the effect of inbreeding on fat production. Differences among the individual sires' regressions were not significant. Inbreeding affects production mostly through channels other than reducing general size. This was shown by comparing the regression of production on inbreeding with and without holding heart girth constant. The partial regression was still about three-fourths as large as the simple regression. Partial regression of production on inbreeding, with the dam's production held constant, did not differ appreciably from the regression obtained when the dam's record was disregarded.

In certain situations, inbreeding is an unavoidable consequence of the breeding plan. Among these would be linebreeding programs, and when herds are thought to be of such high genetic merit that breeding animals of higher merit can be found within the herd than in other herds. The primary purpose of the present study was to assess the effect of inbreeding on production, so as to estimate whether the loss (if any) in average phenotypic merit, because of the inbreeding, is likely to exceed the gains which are sought in the plan.

Woodward and Graves *(12, 13)* and Swett *et al. (10)* reported on the effect of inbreeding on production, in the experiment started by the USDA to determine if farmers' herds could be upgraded by using only one registered sire in a sire-daughter mating system. In the first few generations, the inbreeding effects were confounded with breed differences. However, after the cows became predominantly one breed, further inbreeding caused a decrease in milk and butterfat production.

Bartlett and Margolin *(1)* compared 39 outbred and 40 inbred Holstein cows and found that the outbreds were significantly higher in milk and butterfat yield, but the inbreds averaged 0.05% higher in butterfat test. Plum *(8)* found a significant intra-sire correlation of -0.24 between inbreeding and butterfat production in a Jersey herd of 181 cows.

The several experiments which were analyzed by calculating intra-sire regressions of production on inbreeding are summarized (Table 1). The regressions by Tyler *et al. (11)* were calculated with the dam's record held constant.

Received for publication August 13, 1957.

[1] Journal Paper No. J-3242 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1053. This project is part of the North Central Regional Project NC-2.

TABLE 1

Average changes in production for each increase of one per cent in inbreeding

Authors	Type of record	Cows	B.F.	Milk	Fat
		(No.)	*(lb.)*	*(lb.)*	*(%)*
Davis *et al. (3)*	305-2×-ME	630	−0.66	− 30.8	+0.003
Laben *et al. (4)*	305-2×Jr2	164	−4.88	−209.8	+0.008
Laben and Herman *(5)*	305-2×-ME	299	−2.07	− 66.1	+0.003
Nelson and Lush *(7)*	305-3×-ME	156	−4.5		
Robertson *(9)*	305-2 yr. 10 mo.	80		− 30.0	0.0
Tyler *et al. (11)*	305-2×-ME	89	−2.32	− 73.8	+0.005

SOURCE AND ADJUSTMENT OF DATA

The data came from the Iowa State College Holstein herd from 1930 to June of 1955. After 1930, the herd was almost closed to outside breeding. No outside bulls at all were used after 1934 and no females were introduced after 1937. The purpose of closing the herd was to determine the effects of selection, combined with such inbreeding as would necessarily arise in a finite population of this size. In the selections, production was considered roughly three times as important as type, and information on relatives was given some attention in the selection index. In the earlier years of the experiment, the selection differential for females could not be high because of many troubles, such as pneumonia and reaction to Bang's tests. Sons of the best cows were picked for sires and, as soon as they were of serviceable age, were bred to approximately 40 cows, then were sold with the option of repurchase. This option was exercised only twice.

The 534 cows with records were daughters of 69 sires. Among them they had 1,350 production records averaging 414 lb. of butterfat. The records were standardized to a 305-day, two-time milking, mature equivalent basis.

Most of the inbreeding was slight. The coefficients (Wright's) ranged from 0 to 34% with a mean of 7.4 and a standard deviation of 6.4. The average inbreeding of the cows which calved in 1954 was 11.8%. During the early years of the experiment only a few were inbred at all, and few of these more than 12.5%. In later years, as the background relationship became higher, the average inbreeding increased and the distribution of inbreeding coefficients became less extremely skewed, but the long tail of the curve remained in the direction of high inbreeding, because the bull in service could not have more than one dam and rarely had any full-sisters among his mates.

METHODS AND RESULTS

Simple regressions of production on inbreeding. An inbreeding experiment concerned with dairy cattle is necessarily a long-term project. This is especially so when there is no special effort to increase the inbreeding rapidly. The main reason for this is that the generation interval for dairy cattle is of the order of five years. One cannot hope to hold environmental conditions entirely constant during so long a period as this experiment. Even if it had been physically and

economically feasible to control feeding and management conditions, other extraneous factors, such as temperature, pasture quality, and incidence of disease, are impossible to control wholly. Some important effects of these may even be unknown. Furthermore, the mean genetic composition of the herd can scarcely have remained constant during the past 25 years. The selection for production is likely to have made some genetic changes, natural selection may have been effective in unknown ways, and the very nature of inbreeding permits gene frequency to drift randomly. These sources of change are in addition to any general effects of the inbreeding. The fact that more of the less inbred cows made their records in the earlier part of the experiment, whereas the more highly inbred cows were more frequent in the later years, confounds trends in average inbreeding with time trends in other factors.

Making the analysis on an intra-sire basis would bypass most of the effects of both genetic and environmental time trends, by removing the variance caused by trends and leaving that variance unanalyzed. This is because the comparisons then are only between daughters of the same sire. Since the sires in this herd were nearly always used for only about a year, their daughters were likely to have been nearly contemporary in their lives and production. The pooled intra-sire regression of production on inbreeding is a weighted average of the individual regressions for the daughters of the several sires.

The assumption, usual in the case of regressions, that for complete validity the X's (the independent variable) are measured without error, need not be fulfilled in this case. The X's used in the calculations are inbreeding coefficients calculated according to Wright *(14)*. These are merely expectations of the probable loss in heterozygosis and, therefore, could be in error for any one animal because of the random chance in Mendelian segregation and recombination. However, such an error is merely the amount by which actual heterozygosis is more or less than the computed X. Provided that the only reason for there being any regression at all of production on inbreeding is the inbreeding itself, the true value of Y is correlated with these errors in X. This introduces a term in the numerator of the regression which cancels the effect of the denominator being too large. Berkson *(2)* has advanced a more comprehensive explanation of the reason the regression is unbiased in such cases. Some slight error in the independent variable could arise if differential mortality between the inbreds and noninbreds caused the actually more heterozygous animals to be more likely to live to production age than the less heterozygous ones, which had the same inbreeding coefficients. This would tend to make the regression coefficient a bit nearer to zero than it should be.

TABLE 2

Intra-sire correlations and the regressions of production on one per cent of inbreeding

	B.F.	Milk	Fat
			(%)
Regression	-1.74 ± 0.57	-54 ± 17	$+0.003 \pm 0.003$
Correlation	-0.14 ± 0.05	-0.15 ± 0.05	$+0.05 \pm 0.05$

All of the 534 cows were used in this part of the study. The simple intra-sire regressions and correlations are shown (Table 2). The correlations between inbreeding and production characters would all have been slightly smaller if single records instead of averages had been used.

The present estimates seem low, compared to the estimates Nelson and Lush *(7)* found, using part of these same data. The population would have had to behave quite differently since 1942, because there would certainly be some automatic resemblance in the regressions. To inquire into this, the data were divided into two parts: cows born before 1940 and cows born after 1940. The within-sire regressions of butterfat yield on inbreeding were then calculated for both periods. The regressions calculated for cows born before 1940 were not expected to correspond exactly to the figure from Nelson and Lush. It was not possible to use precisely the same cows and there were some differences in the records used. For instance, their data were on a three-time milking basis. The regression found here for cows born before 1940 was −3.89 lb. of butterfat and for cows born after 1940, −1.33 lb. The difference between the two regressions was not significant; however, it was nearly so ($t = 1.68$ with 462 degrees of freedom). If the regression from Nelson and Lush (−4.5 lb.) is corrected approximately for three-time milking by subtracting 20%, it becomes −3.6 lb.

Effect of inbreeding with dam's record held constant. Morley *(6)* suggested that the simple linear model, such as was used in the previous section, might be inadequate when the population had been subjected to selection. He reasoned that sires would be selected from the genotypically superior female families. The sires, thus being more closely related to the females in these superior familes than to the rest of the herd, would tend to produce their more highly inbred offspring from females above average in transmitting ability. If this bias were important, the simple regression model would underestimate the real effect of inbreeding. The bias will be more when the heritability of the character concerned is high. The extent of this bias will depend on several things, especially on the family structure of the herd and whether the sires are, in fact, much more closely related to the higher-transmitting half of the cows than to the lower-transmitting half. The matings in the Iowa State College herd were made without any conscious attempt either to avoid or to favor inbreeding.

Morley recommended that the phenotype of the dam be considered as an additional independent variable. Then an intra-sire partial regression of production on inbreeding should give an estimate unbiased by any average relation between production of dam and inbreeding of daughter.

Only 484 cows were available for this part of the analysis, because some cows used in the simple regressions had no record available for their dams. This analysis was also on an intra-sire basis.

The correlations between dam's production and daughter's inbreeding are all very small. Only the one for butterfat percentage approaches significance. It is the smallness of these correlations which makes the partial and simple regressions so nearly alike and keeps Morley's argument unimportant in these data.

TABLE 3
Effect of inbreeding with and without considering dam's record

	Regression of daughter's production on each per cent of her inbreeding		Correlation between dam's production and daughter's inbreeding
	Partial	Simple	
Butter yield	-1.72 ± 0.57	-1.72 ± 0.58	-0.001 ± 0.05
Milk yield	-52 ± 17	-50 ± 19	0.05 ± 0.05
Butterfat percentage	0.003 ± 0.002	0.002 ± 0.003	-0.09 ± 0.05

Effect of inbreeding with heart girth held constant. According to Nelson and Lush *(7),* increased inbreeding caused a decrease in the cow's size in the Iowa State herd. They found that size was affected most at 2 yr. of age and that the effect decreased as the cows got older. At 5 yr. of age, the regression of size on inbreeding was positive. They postulated that inbreeding slowed down the growth rate but did not limit utimate mature size. However, their data at the older ages were few. In the present analyses, it was thought desirable to find whether the depressing effect of inbreeding on production was due to decreased size of the more highly inbred cows, or if inbreeding was acting on production through channels other than its effects on size.

First records were used for this part of the analysis. When the cows started their first production records, their ages ranged from 21 to 36 mo. The records were converted to maturity, in order to eliminate differences caused by this range in ages. The cows were measured at as nearly 2 yr. of age as was practical. Seldom did the cow's age at measurement deviate more than 1 wk. from its second birthday. Measuring the cows at a fixed age, while they naturally varied in their age at first calving, allowed differences in the stage of gestation to introduce some extraneous variation in the measurements. Heart girth was used because it was thought to be the best measure of general size (among those measurements available) and was not likely to be influenced much by stage of gestation.

Multiple regression (intra-sire) was the method of analysis, just as in studying the dam's production. The 495 cows which also had a 2-yr.-old heart girth measurements were the progeny of 54 different sires. This and subsequent analyses were confined to butterfat production, because inbreeding seemed to have little or no effect on butterfat percentage.

The simple regression of production on inbreeding was -2.72 ± 0.63 lb. of butterfat; whereas, the partial regression of production on inbreeding with heart girth held constant was -2.05 ± 0.64 lb. The simple regression of heart girth on inbreeding was -0.30 ± 0.05 cm. for each increase of 1% in inbreeding. The simple correlation between heart girth and butterfat production was $+0.22 \pm 0.05$. All of these regressions and correlation coefficients are significantly different from zero at a probability level of less than 1%. The difference between 2.72 and 2.05 seems to indicate that the depressing effect of inbreeding on production is in part a consequence of its having depressed size or growth rate,

but the 2.05 indicates that much of its effect on production goes through one or more other channels.

Test for linearity and homogeneity of regressions. It seemed desirable to investigate whether the regression of production on inbreeding is, in fact, linear. If all the effects of the genes were simply additive, there is no apparent reason for inbreeding per se to have any effect on production. If all of the nonlinear effects of the genes are those due to some degree of dominance of genes for high production (even including overdominance), inbreeding would cause production to decrease, but the decrease should be linear with the inbreeding coefficient. Certain kinds of epistatic effects would cause a curvilinear decrease in production. For instance, those epistatic effects which involve the simultaneous presence of the heterozygote dominance deviations in at least two different pairs of genes would cause a curvilinear decrease. The more pairs of genes involved in such combinations, the faster inbreeding would break up the combinations. If inbreeding reached a level of 50%, theoretically only 1/16th of the 4-pair, one-eighth of the 3-pair, one-fourth of the 2-pair, and one-half of the 1-pair combinations would be left.

The regressions presented in the previous sections were average regressions for all sires. It seemed appropriate to find whether the regression coefficients of individual sires differed from each other more than would be expected by chance. Such differences might be expected if the inbreeding decline were due only to the uncovering of recessives and the sires varied much in the number of these they carried. To test these two things, the analysis of variance (shown Table 4) was calculated. In order to get a reasonable number of cows within

TABLE 4

Analysis of variance to test for linearity of the regressions and for homogeneity of the regressions of individual sires

Variation due to:	Sum of squares	Degrees of freedom	Mean square
Total	2,172,262	501	
Among sires	419,599	44	
Within sires	1,752,663	457	
Among F_x classes within sires	503,576	157	3,207
Pooled intra-sire regression	45,951	1	45,951**
Among regressions of individual sires	91,608	44	2,082
Deviations from linear regressions	366,017	112	3,268
Within F_x classes within sires	1,249,087	300	4,164

** Statistically significant beyond the .01 level.

each inbreeding class, the cows were groped in intervals of 3% of inbreeding. Sires with only one or two daughters were discarded, because they did not contribute to the analysis. This left 502 cows by 45 sires. Again, the average of all of a cow's lactations was used as a measure of its butterfat production.

The surprisingly small mean square for differences among the regressions of individual sires seems to indicate that the decline in production caused by inbreeding is due to many genes, each of which would necessarily have a rather small effect. If this decline were due to inbreeding uncovering a very few major recessives with important detrimental effects, surely some of the sires would

have been heterozygous for more of these major genes than others. Thus, differences among sires should have been found. The smallness of this mean square also appears to indicate that the variation among sires in transmitting ability was small. A positive correlation between the inbreeding of a sire's daughters and their relationship to him is a necessity. Then, if a sire had an individual transmitting ability far above the level of the herd, his inbred daughters, being more closely related to him, should for that reason have higher production. Conversely, if some sires with transmitting ability far below average were picked by mistake, their more highly inbred daughters should be farther below the herd average.

The small mean square (3268) in Table 4 for deviations from linearity for each individual sire's regression line indicates that the true regressions are linear. Otherwise, this mean square would be expected to be larger than the mean square just below it. The data actually agree a little better than expected with the hypotheses that the regressions for individual sires are *(a)* equal and *(b)* linear.

DISCUSSION

The level of inbreeding reached in this herd, after more than 20 yr. essentially without introduction of outside blood, emphasizes that a herd of this size can be closed for a long time without the inbreeding reaching a high level. Actually, since production has been increased above starting levels, the accumulated inbreeding might be considered as an additional benefit, since that gives some promise that the cows would transmit their high level somewhat more uniformly. If the number of sires in use at any one time had been still larger, and an effort had been made to avoid mating highly related animals, the level of inbreeding could have been kept even lower.

Intra-sire regressions of −1.74 lb. of butterfat, −54 lb. of milk, and +0.003% butterfat, for each increase of 1% of inbreeding, are well within the range of the findings reported by other workers. Most of the investigations, including this one, have found a slight positive regression of butterfat per cent on inbreeding. It is well known that environmental conditions which affect milk and butterfat production adversely tend to raise butterfat percentage. Perhaps this is enough to explain the apparent slight effect of inbreeding on butterfat percentage.

Although not quite significant, the difference between the regressions for cows born before and after 1940 seems unduly large. There is approximately only one chance in ten that a difference this large would have been found, if, in fact, there were no true difference. Selection has been much stronger since 1940 than before, and it seems plausible that this might have had some influence on the effect of inbreeding. If the regression were curvilinear, there could be a difference in the two linear regressions, because the more highly inbred cows tended to make records after 1940. However, no evidence was found to indicate that the regression was not linear.

Holding the dam's record constant did not alter the regression coefficients appreciably. This happened because the correlations between the dam's pro-

duction and daughter's inbreeding were small and varied in signs. Considering that approximately one-tenth of the data had to be discarded, and that the simple and partial regressions were nearly alike, holding the dam's record constant seems not to have added enough information to be worth while in these data.

Reduction of the simple regression of -2.72 lb. to -2.05 lb. for the partial regression of inbreeding on first lactation fat production, when heart girth is held constant, indicates that inbreeding causes some of the decrease in production through its detrimental effect on body size. This would be expected from the a priori knowledge of the positive correlation between size and production and the negative correlation between inbreeding and size. Although this reduction is only of the order of one-fourth, perhaps it should be considered when assessing the effect of inbreeding on production, because the smaller cow is not necessarily less economical even though on the average it produces less.

A difference as large as the one between the regression of -2.72 for first records and -1.74 for average records seems too large to be written off as likely to have been caused by chance alone. A plausible explanation of the differences between the regressions for first and average records is that the depressing effect of inbreeding on size and production is materially reduced at older ages; i.e., that inbreeding acts more to lower the rate of development than it does to limit eventual size and production at maturity. Therefore, when average records are used some, but not all, of the adverse effect of inbreeding on production would be removed.

It would be interesting to know whether the effect of inbreeding on production is reduced or eliminated at maturity. Unfortunately, when the population has been subjected to selection, no way to evaluate the effect of inbreeding at different ages is apparent. If the cows are selected on their own performance (as was the main basis for their selection here), the older cows would tend to be the better producers, regardless of their inbreeding coefficients.

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PERMANENCE OF TYPE RATINGS IN YOUNG HOLSTEIN HEIFERS[1, 2]

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SUMMARY

To determine the repeatability of type ratings in young heifers, 544 animals in five herds were evaluated at 3, 6, and 12 mo. of age. Evaluations were made on a ten-division score-card basis, and each animal was scored independently by two evaluators working simultaneously. Repeatabilities of the various type components were estimated by intra-heifer, intra-herd correlations. The correlation values between 3- and 6-mo. scores ranged from 0.2 to 0.3 for the different score-card divisions. Values between 6 and 12 mo. showed a somewhat wider range, and those between 3 and 12 mo. were somewhat lower. Variations among herds and among score-card divisions within herds are a definite limitation upon the usefulness of this information in small groups of heifers. Definite, characteristic age changes were observed in all score-card divisions. The most consistent of these were a progressive improvement in shoulders and chest (more marked between 3 and 6 mo., but also significant between 6 and 12 mo.), a drop in scores for rump and thigh between 6 and 12 mo., and a marked improvement in feet and legs between 3 and 6 mo. With some exceptions, there was a rather strong tendency for heifers to be scored higher at 6 mo. of age than at other ages. This was particularly noticeable in total score.

Since type classification has been in general use, the permanence of such ratings has been of considerable interest. Most of this interest has been in cows of producing age. The possibility of phenotypic and genetic correlations between type and production has also been investigated. Although type in heifers has received some attention, no study has been reported in which the heifers were evaluated at specific ages. In most studies, type has been evaluated only in rather broad, general terms, most of the interest being in a single, final rating.

The present study was designed to evaluate the changes in body conformation of the young heifer, by scoring type in considerable detail at specific ages. Some of the changes which occur between 3 and 12 mo. of age are discussed.

Johnson and Lush *(7)* found the intra-cow correlation between different classification ratings to be 0.55 when the ratings were made by a three-man committee, and 0.34 when ratings were made by individual judges. Hyatt and Tyler *(5)* found the intra-cow, inter-judge correlation between different ratings in one Ayrshire herd to be 0.55. Benson *et al. (1)* estimated the repeatabilities of the ten divisions of the Ayrshire score-card to range from 0.3 to 0.6 for cows. Similar repeatabilities for heifers ranged from 0.0 to 0.3. Hyatt *et al. (6)* found the regression of average cow rating upon average heifer rating to be 0.45.

Received for publication January 12, 1957.

[1] This study was made through the cooperation of the Dairy Husbandry Research Branch, in the cooperative project, "Improvement in Dairy Cattle Through Breeding."

[2] These data were taken from material submitted by the senior author in partial fulfillment of the requirements for the Doctor of Philosophy degree, The Ohio State University, Columbus, Ohio.

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Harvey *et al. (4)* concluded that type changes in heifers between 6 and 24 mo. were largely unpredictable.

METHODS

Five hundred and forty-four Holstein heifers in the Ohio NC-2 Breeding Project were evaluated for type at 3, 6, and 12 mo. of age. This group included all of the heifers raised through these ages during a three-year period ending February, 1954, in five herds owned by the Ohio Department of Mental Hygiene and Correction. These herds, and the number of heifers in each, are: Athens State Hospital, 72; Dayton State Hospital, 107; London Prison Farm, 158; Orient State School, 73, and Toledo State Hospital, 134. These herds are visited bimonthly on approximately the same date each time, and the heifers are evaluated at the time of the visit nearest the selected ages. With a few exceptions, the evaluations used are the averages of the scores of two judges working independently at the same time. Four different judges did appreciable amounts of the work on the Toledo herd, but most of the evaluations in the four other herds were made by the same two men. An Ayrshire score-card was used, slightly modified. The judges worked together in several practice sessions, to standardize their interpretations of the various score-card divisions. The designations of the last two divisions were changed from general quality and breed character to dairy character and general appearance, because these terms seemed to be more descriptive. Other divisions may not have been defined exactly as they were originally intended. The final, or total, score is the sum of the ten division scores. Special emphasis was placed upon rating each score-card division as objectively as possible, without reference to any preconceived idea of over-all rating or total score.

These data were analyzed for repeatability and age differences.

RESULTS AND DISCUSSION

Since herd differences in average score were apparent, repeatability was first estimated within herds. These five estimates were then combined by converting each r to weighted z, Snedecor *(9)*. The herd differences in repeabability were tested for significance, and an average r was computed for each division in which the herd difference was not significant at the 1% level of probability. These results are summarized (Tables 1, 2, and 3). In the 3 to 6 mo. comparison, the difference in repeatability was obviously due to the low r in the London herd. In the two other age comparisons (6-12 mo. and 3-12 mo.), significant differences were found among herds in the repeatability of scores for size and placement of teats. These differences could not be attached to particular herds. Finally, the data from all herds were pooled, to give a single estimate for each score-card division. In general, this latter method yields estimates which are equal to or higher than those derived from the average z's. The lower r's for udder size and shape in both the 3-6 and 3-12 mo. comparisons are notable exceptions. In this division, different herds appear to have conflicting trends between three mo.

TABLE 1

Repeatability of type evaluations from 3 to 6 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.379	0.303	0.268	0.570	0.385	0.460	0.351	0.216	0.551	0.470	0.475
Dayton	107	0.301	0.411	0.319	0.256	0.441	0.183	0.080	0.289	0.364	0.350	0.466
London	158	0.214	0.101	0.301	0.154	0.272	0.300	0.342	0.446	0.146	0.144	0.079
Orient	73	0.170	0.341	0.316	0.394	0.364	0.174	0.091	0.453	0.268	0.248	0.357
Toledo	134	0.280	0.280	0.292	0.342	0.235	0.174	0.184	0.127	0.312	0.350	0.321
All	544	0.313	0.393	0.320	0.335	0.468	0.143	0.242	0.282	0.333	0.378	0.445
Average		0.263	0.267	0.301	0.313[a]	0.325	0.253	0.223	0.317[a]	0.305[a]	0.296	[b]

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 2

Repeatability of type evaluations from 6 to 12 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.370	0.217	0.181	0.466	0.307	0.248	0.328	0.345	0.359	0.113	0.326
Dayton	107	0.393	0.376	0.032	0.390	0.426	0.309	0.301	0.261	0.125	0.186	0.410
London	158	0.187	0.306	0.221	0.378	0.187	0.144	0.258	0.484	0.279	0.222	0.281
Orient	73	0.211	0.309	0.317	0.368	0.147	0.009	0.163	0.307	0.014	0.255	0.289
Toledo	134	0.255	0.303	0.256	0.297	0.054	0.144	0.134	0.025	0.331	0.342	0.110
All	544	0.295	0.378	0.260	0.410	0.350	0.466	0.466	0.445	0.274	0.427	0.516
Average		0.272	0.308	0.201	0.372	0.216[a]	0.174	0.234	[b]	0.240	0.239	0.273

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 3

Repeatability of type evaluations from 3 to 12 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.032	0.366	0.054	0.345	0.209	0.167	0.336	0.058	0.407	0.149	0.234
Dayton	107	0.176	0.171	0.275	0.158	0.246	0.024	−0.017	0.081	0.190	0.006	0.202
London	158	0.178	0.012	0.072	0.202	0.156	0.318	0.267	0.423	0.231	0.169	0.207
Orient	73	0.248	0.361	0.388	0.366	0.293	0.283	0.293	0.346	0.274	0.117	0.395
Toledo	134	0.130	0.140	0.241	0.049	−0.077	0.028	0.014	0.043	0.129	0.279	0.039
All	544	0.201	0.266	0.222	0.216	0.292	0.102	0.205	0.203	0.277	0.245	0.327
Average		0.157	0.171[a]	0.197	0.198	0.180	0.167[a]	0.171[a]	[b]	0.228	0.157	0.196

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 4

Effect of age on type ratings of heifers

Age (mo.)	Head and neck	Shoulders and chest	Middle loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
	Mean scores										
3	81.6a	80.4a	82.4a	81.6a	79.6a	81.2a	81.2a	81.5a	82.1a	81.4a	812.8a
6	81.6a	81.4b	83.6a	81.8a	80.4b	82.1b	81.9b	82.1b	82.7b	82.2b	819.8b
12	80.8b	81.8c	82.9b	79.9b	80.6b	81.5c	81.6b	81.4a	82.2a	81.2a	813.9a
	Analysis of variance										
	Mean square										
Source											
Ages	116[b]	298[b]	191[b]	614[b]	159[b]	122[b]	146[b]	588[b]	66.5[b]	168[b]	7715
Herds	139[b]	336[b]	98.0[b]	124[b]	624[b]	476[b]	577[b]	40.8[b]	88.5[b]	544[b]	23985
a × h	11.4[a]	14.9[b]	9.75	16.5[b]	9.12	144[b]	67.9[b]	51.1[b]	5.09	24.4[b]	1150.5
Error	4.58	5.27	5.64	5.57	7.72	4.86	5.48	4.45	3.66	5.49	160.54

a, b, c—Age means in each score-card division which are followed by the same letter are not significantly different; means followed by different letters are significantly different. The methods described by Duncan *(3)* were used to test these differences.

[a] Difference between herds significant at the 5% level.

[b] Difference between herds significant at the 1% level.

and the other ages. In some instances, the combined data probably yield a more generally applicable estimate of repeatability. However, any environmental influences which affect type scores contribute toward higher estimates, and this fact should be recognized. The estimates derived from the average 2's appear to be more conservative.

Age differences in the data were tested for significance by analysis of variance. These results are summarized (Table 4). The differences between ages and differences between herds were significant well beyond the 1% level of probability in each year-and division. This was not true, however, for all divisions for age / herd interaction. The apparent disagreement with the findings of Harvey et al. (4) is not considered serious in view of the difference in age of heifers studied, the age-grouping procedure of those workers, compared with the exact ages (= above term) days, in the present study, and the much more detailed year-and used here, in contrast to the more general year-and divisions used by the Idaho workers.

The most consistent age changes were found in shoulders and chest, rump and thighs, and feet and legs. Scores for shoulders and chest tend to improve progressively from 3 to 12 mo. of age, but the greatest improvement is made before the animals are 6 mo. of age. Each herd exhibits the same trend, except the Toledo herd, which shows no improvement between 6 and 12 mo. A possible explanation of this improvement is suggested in unpublished data by Clifton and Richard (2), which show that the spinal processes usually grow above the top of the shoulder blades during this age period. Of 256 heifers in three herds, only ten had spinal processes above the shoulder blades at 3 mo. of age. The processes began to protrude between 3 and 6 mo. on 125 heifers, between 6 and 12 mo. on 76, and had not protruded at 12 mo. on 27 heifers. The remaining 16 heifers showed inconsistent change. The protrusion of these processes, no doubt, make the withers appear sharper and the shoulders more nearly arranged.

Scores for rump and thighs drop markedly between 6 and 12 mo. This is in apparent agreement with the findings of Leighton and Graves (6), who found that rump slope increased with age.

Feet and legs show significant improvement between 3 and 6 mo., but the Dayton herd shows marked improvement between 6 and 12 mo. Since the heifers in this herd are somewhat retarded in growth, an environmental effect is suggested.

Other age changes which appear to be consistent in most herds are a drop in head and neck scores between 6 and 12 mo.; an increase in scores for middle and loin between 3 and 6 mo., followed by a downward trend at 12 mo., and a general tendency for heifers to score higher at 6 mo. in all udder divisions, dairy character, general appearance, and total score. Herd differences, particularly in the last two, seem to reflect environmental differences, and are greatest at 12 mo. of age. Herd differences were also quite apparent in the scores of the udder divisions. Although environment may account for some of the herd differences, it does not seem to furnish adequate explanations for all differences. The erratic repeatability estimates in these divisions can be explained by these herd differ-

ences in age trends. In addition to herd differences, certain heifers exhibit marked departure from the general trend. The possibility that certain related groups within herds may exhibit their own characteristic trends is suggested.

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BACTERIOLOGICAL STUDIES OF UTERI OF CLINICALLY NORMAL COWS OF LOW FERTILITY[1]

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SUMMARY

This study was undertaken to determine if the uteri of clinically normal repeat-breeder cows contained microorganisms at 16 days postbreeding, a stage immediately followed by a high incidence of embryonic death. Sixty-nine repeat-breeders were slaughtered, the uteri removed aseptically, and flushed with 15 ml. of 0.1% tryptone broth. Various media were inoculated with the recovered fluid and incubated in air, in 15% CO_2 and reduced air, and in an anaerobic jar. Triturated endometrial tissue of 35 animals in the study was similarly cultured. Microorganisms were recovered from the uteri of eight (11.6%) of the 69 repeat-breeders. A yeast, *Candida krusei,* was isolated from four uteri, an *Escherichia* species from one, a *Pseudomonas* from one, *Brucella abortus* from one, and a paracolon from one. Embryos were recovered from 31 of 62 uteri from cows with anatomically normal reproductive tracts; three embryos were recovered from the eight infected uteri. Vaginal mucus samples were obtained from 35 of the animals at from 11 to 13 days postbreeding and at slaughter. One cow had a positive mucus agglutination test for *Vibro fetus.* Serum agglutination-lysis tests for leptospirosis were made on all animals. None was positive. Microbial infection apparently accounted for few of the embryonic deaths in these clinically normal repeat-breeders.

Embryonic death is a major cause of infertile services in dairy cows of low fertility; this is especially true in those animals that show no recognized clinical manifestations for which known therapy is indicated *(2, 5, 13, 14)*. It appears that the majority of these deaths in such repeat-breeding cows occurs shortly after the 16th day of gestation *(5)*. The presence of low-grade infections, for which characteristic pathology is not detected, has frequently been proposed as the cause of early embryonic mortality in these cows. Infusion of the uteri with antibiotics at the time of, or immediately prior to, breeding, however, did not lessen the embryonic death *(15)*. But the apparent time of death of the embryo in these cows (16 + days) might be the result of infectious agents entering the uterus after the time of treatment. Contradicting the work of Ulberg *et al. (15)*, Lindley *(9)* has reported considerable increase in the conception rate of repeat-

Received for publication July 24, 1957.

[1] Paper No. 652, Department of Genetics, University of Wisconsin, and Paper No. 239 NS, from the Department of Veterinary Science. Published with the approval of the Director of the Agricultural Experiment Station. This work was done under a cooperative agreement between the Wisconsin Agricultural Experiment Station and the Dairy Husbandry Research Branch, USDA. It has been supported in part by a grant from the Research Committee of the Graduate School, from funds supplied by the Wisconsin Alumni Research Foundation and by grants from Badger Breeders' Cooperative, Consolidated Breeders' Cooperative, East Central Breeders' Cooperative, Southern Wisconsin Breeders' Cooperative, and Tri-State Breeders' Cooperative; the experimental cows were also secured through their help.

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breeding cows, with apparently low-grade infections, by intra-uterine infusion of antibiotics and sulfonamide solutions one or two days following service.

The possible role of microorganisms in the repeat-breeding problem has received considerable attention and has been reviewed extensively *(1, 10)*. More recently, much attention *(3, 11)* has been paid to *Vibrio fetus* as a cause of lowered fertility in the repeat-breeding dairy cow. In a recent extensive study, Gunter *et al. (4)* demonstrated the presence of pathogenic micrococci, diphtheroids, and streptococci from the uteri of repeat-breeding cows; whereas, from normal-breeding animals predominantly saprophytic strains of bacteria were obtained. Furthermore, Gunter *et al. (4)* report that 38% of the samples from the uteri of normal cows was sterile; whereas, only 5% of similar samples from repeat-breeders was sterile.

Our present study was undertaken to determine the percentage of repeat-breeder cow uteri containing microorganisms at 16 days postbreeding, an estimate thus being obtained of the importance of infectious agents in the uterus as causes of the embryonic death occurring shortly after 16 days. The assumption was made that microorganisms causing subsequent embryonic death would likely be present in the uterus at the time of slaughter.

MATERIALS AND METHODS

Selection and handling of cows. Sixty-nine cows of low fertility were used in the study. They were obtained between April, 1955, and March, 1956, from farms throughout Wisconsin and east-central Minnesota and were selected on the following bases: *(a)* Bred at least four times without apparent conception, *(b)* bred last within the past 90 days and returned to heat, *(c)* no purulent discharge, *(d)* no abnormality of the reproductive tract detectable by palpation per rectum, *(e)* not more than ten years old, and *(f)* Bang's-negative. One cow was obtained from each of 56 herds, two cows from each of four herds, and five cows from one herd.

The 69 experimental repeat-breeders included Holstein, Guernsey, Jersey, and Brown Swiss dairy cattle. Fifty-five had calved at least once and 14 were nonparous heifers. Each had been bred from four to 21 times before the experimental service; the mean was 7.2 services. All except a very few of the services on the farms were by artificial insemination, with semen diluted with milk or egg yolk–citrate and containing antibacterial agents.

The cows were trucked to a University barn near Madison as they were selected. Heat-checks were made twice daily, a cow being considered in heat when it stood for mounting by other cows. The animals were artificially inseminated approximately 12 hr. after estrus was detected, with 1 ml. of day-of-collection semen diluted with milk or egg yolk–citrate and containing sulfanilamide, penicillin, and streptomycin. The semen was obtained from unselected stud bulls used regularly in artificial breeding, and was deposited in the body of the uterus. Two heifers failed to show signs of estrus in connection with ovulation, soon after being brought to the experimental barn. They were slaughtered

15 days after the time of ovulation, estimated from the routine sequence of palpation of the ovaries per rectum.

The reproductive organs were palpated at least once between eight and 15 days postbreeding, to determine the presence of a corpus luteum and the tone of the uterus. The majority of animals were slaughtered at the end of 16 elapsed days from the beginning of heat; three were slaughtered at 17 days, and 19 animals that were first noticed in heat in the evening were slaughtered 15⅔ elapsed days after the first signs of estrus.

The animals were slaughtered at the University meats laboratory. After being stunned, each was bled while lying on its side rather than while hanging from a hoist. This procedure was followed to reduce the possibility of bacterially contaminated vaginal mucus draining through the cervix into the uterus. Immediately after the animal was dead, it was rolled on its back and the abdomen opened. The uterine horn on the side opposite the ovary which contained the corpus luteum was ligated near the tubo-uterine junction, to prevent possible drainage through the oviduct of peritoneal fluid which might have been contaminated when the peritoneal cavity was opened. The ovaries and reproductive tract anterior to the vagina were then removed from the animal. Vaginal mucus samples were obtained from the last 35 animals on the project; the ventral pelvic bone was cut in these cattle, to facilitate removal of a greater length of the vagina. A ligature was placed around the vagina 4 to 6 in. posterior to the cervix before removal of the reproductive tract, to prevent the escape of vaginal and cervical mucus. The organs were handled throughout in such a manner as to minimize the chances of drainage from the cervix and vagina into the uterus.

The genital organs were examined carefully for any anatomical abnormalities which might have interfered with fertilization. The two uterine horns were then separated by dissecting the intercornual ligament between the external and internal bifurcations; both uterine horns were ligated at the internal bifurcation and excised from the remainder of the tract. The time interval from the stunning of the animal to this point was approximately 15 min.

The uterine horn on the side opposite to that of ovulation was used for the bacteriological study; the other uterine horn was flushed to determine the presence of an embryo. It was assumed that any microorganisms present would likely be in both uterine horns, rather than restricted only to the horn on the side of ovulation. The uterine horn which was to be used for the bacteriological study was placed in a sterile container and taken immediately to the Bacteriology Building, and the work there carried on in a sterile room.

LABORATORY WORK

A small area on the uterine serosa was sterilized by searing with a hot spatula, and 15 ml. of 0.1% tryptone broth was injected into the uterine lumen by puncture of the uterine wall in the seared area with a 19-gauge hypodermic needle. The needle hole was closed by searing with a hot spatula, and the uterine horn

manipulated vigorously, to suspend in the solution any microorganisms which might be in the lumen. The fluid was then forced into the tubal end of the horn, an area seared, and the fluid withdrawn by syringe and hypodermic needle for bacteriological study.

Bacteria could possibly live and multiply within the tissues of the endometrium and cause damage leading to embryonic death, without actually coming in contact with the embryo. Such bacteria might be overlooked by culturing only washings from the uterine lumen. In this work, therefore, bacteriological studies were made of triturated endometrial tissue of the last 35 animals. After the tryptone broth was removed, the uterine horn was placed in a sterile box and an area approximately 2 in. long and 1 in. wide at the level of the external bifurcation was seared with a large spatula. The endometrium was exposed by carefully removing the serosa and myometrium in the seared area. All of the endometrial tissue was excised for a length of approximately 2 in. and placed in a Petri dish, where the most vascular caruncular areas and excess connective tissue underlying the endometrium were removed. The endometrial tissue was cut into small pieces with scissors and triturated aseptically in a TenBroeck grinder in 3 ml. of 0.1% tryptone broth. After the tissue was thoroughly ground, the fluid was decanted and used for bacteriological culture.

Various culture media were used to provide a wide range of growth conditions. The media and the reasons for the use of each were:

(a) Blood agar—Difco blood agar base plus 5% citrated bovine blood was used. Onto the surface of the medium was placed 0.1 ml. of the fluid to be cultured and this was spread with a sterile glass rod. This medium was used as one likely to grow such organisms as staphylococci, streptococci, diphtheroids, brucellae, or *V. fetus*.

(b) EMB agar—Difco EMB agar was used and cultures were prepared by the surface-spread technique. This medium was used to detect the presence of coliforms and related pathogens.

(c) Albimi brucella broth—To this medium was added 0.1% agar to make a semisolid nutrient solution that is known to support the growth of the brucellae and *V. fetus*. This was inoculated with 0.1 ml. of fluid (obtained as just described).

(d) Thiol medium—This Difco medium was supplemented with 0.4% agar and 0.05 g. per liter of glutathione (reduced), according to the method of Hughes and Gilman *(7)*. This medium was inoculated by stabbing it with a loopful of fluid, and was used as one likely to obtain growth of *V. fetus* and anaerobes.

(e) PPLO medium—Difco PPLO agar supplemented with 1% Bacto-PPLO serum fraction was used to prepare plates for the possible detection of pleuropneumonia-like organisms. This medium was inoculated by spreading 0.1 ml. of the fluid over the surface.

TABLE 1

Media and culture environments used

Medium	Number of cultures per sample in each environment		
	Air	Increased CO_2 Decreased O_2	Anaerobic
Blood agar	3 Fluid[a] 3 Controls	3 Tissue[b] 3 Fluid 3 Controls	3 Fluid 3 Controls
EMB	3 Fluid 3 Controls	3 Fluid 3 Controls	3 Fluid 3 Controls
Albimi brucella broth	3 Fluid 3 Controls	3 Fluid 3 Controls	3 Fluid 3 Controls
PPLO	4 Fluid 4 Controls	.....	.....
Thiol	.....	6 Fluid 5 Tissue	.....

[a] Fluid—those with fluid from uterus.

[b] Tissue—from triturated uterine tissue.

A large number of plates and tubes of each medium was made from the fluids collected from each uterus. The plates and tubes were incubated at 37° C. under three different environmental conditions; i.e., in air, in 15% CO_2 and reduced oxygen, and in an anaerobic jar. In all cases, a large number of uninoculated control plates and tubes were incubated, to insure that any growth obtained from the fluids did not arise as contamination, during preparation of the cultures. From each animal there were 25 plates for cultures plus 22 control plates, and 20 tubes for culture plus 9 control tubes. This distribution is shown (Table 1). In addition to the cultural tests just described, the fluids recovered from the uteri were examined under the microscope by the use of stained smears of the material. Vaginal mucus from the last six cows in the trial was inoculated into a special selective medium, Kuzdas and Morse *(8)*, for *V. fetus*.

A blood sample was taken from each animal immediately prior to slaughter. The blood was allowed to clot, the serum removed and frozen for subsequent agglutinin-lysis test for leptospirosis.

The *V. fetus* tube-agglutination test was performed on two vaginal mucus samples from each of the last 35 animals used in the study. The samples were taken at from 11 to 13 days postbreeding and shortly after slaughter. Each sample was tested with two antigens. The method used in obtaining the vaginal mucus and in performing the tests was essentially that described by Hughes *(6)*. In addition, a sample of the uterine washings of each of the 35 animals was tested in the same manner.

Soon after the animal was slaughtered, the uterine horn on the side of ovulation was flushed with about 15 ml. of physiological saline solution. Each embryo that was recovered was measured, then examined under a low-power microscope for normality and measurement of the embryonic disc.

RESULTS

Organisms were cultured from the uterine washings of eight (11.6%) of the 69 repeat-breeder cattle included in the study. A yeast, *Candida krusei,* was recovered from four of the infected uteri, a paracolon (family *Enterobacteriaceae*) from one, an *Escherichia* species from one, a *Pseudomonas* species from one, and *Brucella abortus* from another, the latter animal being Bang's-negative at the time of purchase.

The experimental animals were classified according to the presence or absence of: (1) mechanical obstructions of the reproductive tract, (2) pus in the uterus at the time of slaughter, (3) an embryo, and (4) isolation of microorganisms from the uterus (Table 2).

Five of the 69 animals had mechanical obstructions or abnormalities of the reproductive organs which might have interfered with fertilization. One animal had a bilateral hydrosalpinx, one had adhesions of the bursa to both ovaries, two had adhesions of the infundibulum to the mesosalpinx, and one had a completely double cervix. None of these five animals had an embryo in the uterus and no microorganisms were cultured from the uterine washings.

TABLE 2

Classification of experimental animals based on the presence[a] or absence of (1) *mechanical obstruction of the reproductive organs,* (2) *pus in the uterus,* (3) *an embryo, and* (4) *microorganisms isolated*

Mechanical obstruction	Accumulated pus	Embryo	Microorganism isolated
5+	0+	.....	.....
	5—	5—	5—
64—	7+	1+	1—
		6—	4+[b]
			2—
	57—	30+	3+[c]
			27—
		25— (plus two not bred)	1+[d]
			26—

[a] The presence or absence of each factor is indicated in the table by a + or — sign. The numeral preceding each sign is the number of animals in the classification.

[b] One isolation each of *Candida krusei, Pseudomonas, Escherichia,* and a paracolon.

[c] Two isolations of *C. krusei,* one of *Brucella abortus.*

[d] *C. krusei.*

The other 64 animals had reproductive tracts which were normal anatomically. Gross inspection of the uterus and observation of the uterine washings

revealed that seven of the 64 had an accumulation of pus in the uterine lumen. The quantity of the pus was in the order of 1 ml. or less in each uterine horn of six of these cows; the uterus of one cow contained approximately 10 ml. of thick, gummy pus in each horn. Neither the quantity of pus in the uterine lumen nor the tone of the uterus was such that the condition could be detected by rectal palpation of the uterus prior to slaughter. An embryo was recovered from one of these seven uteri; the washings from this uterus and from two others which contained small amounts of pus were sterile when cultured. Four of the seven uteri containing pus were infected; *C. krusei* was isolated from one, an *Escherichia* species from one, a paracolon from one, and a *Pseudomonas* species from the uterus containing the large quantity of pus.

Fifty-seven of the anatomically normal uteri showed no macroscopic accumulation of pus. Embryos were recovered from 30 of these uteri, 27 of which were sterile bacteriologically. *C. krusei* were isolated from two of the uteri which contained embryos, and *B. abortus* from one. Embryos were thus recovered from three of the eight uteri from which organisms were isolated. Phagocytized gram-positive cocci were seen in the washings of one uterus which contained an embryo, but the fluid was sterile when cultured. Embryos were not recovered from 27 of the anatomically normal uteri which contained no pus. Two of these animals had not been bred experimentally, and no organisms were cultured from their uterine washings when they were killed approximately 16 days post-ovulation. Of the other 25 uteri from which embryos were not recovered, 24 were sterile bacteriologically, and *C. krusei* was recovered from one. One of the cows whose uterus was sterile bacteriologically gave a positive vaginal-mucus tube-agglutination test for *V. fetus* on both vaginal-mucus samples. Of the 35 animals tested in this manner, this was the only one which gave a positive test. This particular cow was obtained from a herd in which vibriosis has never been recognized as a problem.

In the case of the four uteri from which *C. krusei* was isolated, the organism was seen in large numbers on microscopic examination of the uterine washings of three of the animals.

An agglutination-lysis test for leptospirosis was performed on blood serum from each of the animals. All of these tests were negative. These tests were performed along with tests on serum samples from a Leptospirosis project from which positive samples were obtained. Thus, we were confident that the agglutination-lysis test was working properly.

Fourteen of the repeat-breeders were nonparous heifers. Three of these heifers had mechanical obstructions which might have interfered with fertilization; one had adhesions of the infundibulum to the mesosalpinx, one had ovarian adhesions, and one had a double cervix. These heifers had no embryos. Two of the heifers were those animals which were not bred. Three of the nine other heifers had an embryo in the uterus and six did not. Microorganisms were not recovered from the uterus of any heifer.

The results of this study fail to indicate that infectious agents in the uterus

cause an appreciable amount of the embryonic death which occurs shortly after 16 days postbreeding in the clinically normal repeat-breeder cow. Again, it must be emphasized that this is a selected kind of cow, and that the observations in this study have little bearing on the broad role of microorganisms in lowered fertility in cattle. Most of the studies previously reported either have ignored the clinical variations in the cows under consideration or have intentionally limited the study to clinically abnormal animals. The main reason for the present results differing from others probably rests on this difference in animals.

The possibility also should not be completely ignored that organisms actually were present which were of a kind that produced little or no inflammation [uterine condition described by Simon and McNutt *(12)*] and which could not be detected by our methods. Then, too, the possibility of there being undetermined carry-over effects from earlier infections, which interfered with the physiological ability of the uterus to support a pregnancy, probably deserves some inquiry. Logically, these two possibilities must be kept in mind, in dealing with the general problem. For the present, however, there seems to be little evidence that infectious agents play a major role in the lowered fertility of cows such as studied here.

ACKNOWLEDGMENT

The authors are indebted to Dr. L. J. Wickerham of the Northern Utilization Research Branch, A.R.S., U. S. Department of Agriculture, for the identification of *C. krusei*, and to Drs. S. H. McNutt and J. Simon of the Department of Veterinary Science, and to Dr. L. E. Casida of the Department of Genetics, for their advice and guidance during this work.

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EFFECT OF PREVIOUS RATION ON LACTATION RESPONSE TO SUBSTITUTING CORN FOR ALFALFA ACCORDING TO NET ENERGY AND TOTAL DIGESTIBLE NUTRIENT SYSTEMS OF ESTIMATION[1]

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SUMMARY

The lactation response to the substitution of 10 lb. alfalfa with 7.0 or 5.7 lb. corn based, respectively, on the TDN or NE system was studied, in a double-reversal trial, with 24 cows previously fed equivalent therms NE per unit of live weight from either of two basal rations: 100% alfalfa or 80% alfalfa plus 20% corn. Basing the substitution on the TDN system resulted in greater energy intake than the NE system, 0.99 therm and 0.94 lb. TDN. Across both basal rations, the TDN basis of substitution resulted in significant increases in average daily milk production, 0.89 lb. milk, 0.025 lb. fat, and 0.73 lb. FCM, and a 0.10 decrease in the fat percentage, over the NE basis. Assessing the advantage of the TDN basis of substitution for each basal ration fed, the cows previously fed the 80% alfalfa plus 20% corn basal ration failed to respond; whereas, those previously fed the 100% alfalfa basal showed the following significant advantages: milk 1.56 lb., fat 0.051 lb., and FCM 1.39 lb. When 10 lb. alfalfa were replaced with equivalent NE from corn, milk production increased 1.55 lb. and FCM 0.94 lb. for the 100% alfalfa basal. No significant changes for the 80% alfalfa plus 20% corn basal were observed and across both basal rations, a slight increase in pounds of milk.

The adequacy or inadequacy of an all-alfalfa hay ration for lactation in dairy cattle is controversial. Huffman and Duncan *(14)*, after feeding an all-alfalfa ration, which was considered a "depletion" ration, until milk production and live weight changes became minimal, replaced a portion of the alfalfa with equivalent amounts of total digestible nutrients from corn. Milk production increased and this was attributed to unidentified lactation factor(s) contained in the corn. These same workers, as well as others *(9, 27)*, have obtained similar lactation responses with other concentrates, immature hays, and silages. In contrast, others *(15, 24)*, under somewhat similar experimental conditions, have noted increases

Received for publication July 9, 1957.

[1] This study was made in part with funds provided by the Research and Marketing Act of 1946, through a contract between the Storrs (Conn.) Agricultural Experiment Station and the Dairy Husbandry Research Branch of the USDA, as well as with funds provided by the Chas. M. Cox Co., Boston, Massachusetts. The data are part of a thesis presented by the senior author to the Graduate School, University of Connecticut, in partial fulfillment of the requirements of the M.S. degree.

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in milk production, but accounted for these increases on the basis of greater net energy intake. When net energy was held constant in substituting corn into an all-alfalfa ration, no increase in milk production was observed. Loosli *et al. (19)* essentially have confirmed these observations.

The present study was undertaken to determine the lactation response to the substitution of corn for alfalfa, on an estimated net energy basis and on an estimated total digestible nutrient basis, as affected by previous freeding of equivalent net energy per unit of live weight from either a 100% alfalfa basal ration or an 80% alfalfa plus 20% corn basal ration.

EXPERIMENTAL PROCEDURE

Animals and rations. Twenty-four cows—12 Guernseys, 4 Holsteins and 8 Jerseys—were placed on experiment during the period of January to October, 1953, on either the 14th or 35th day postpartum. Within a breed, groups of four cows were selected for similarity of age and previous calvings (Table 1). Within

TABLE 1

Average age, previous lactations, days in milk, production of 4% FCM, live weight and days in gestation of the experimental groupings with their standard errors

	Basal ration			
	100% Alfalfa		80% Alfalfa + 20% corn	
	Sequence of substitution			
	NE-TDN-NE	TDN-NE-TDN	NE-TDN-NE	TDN-NE-TDN
Years of age at end of depletion period	3.7 ± 0.3	4.0 ± 0.4	3.5 ± 0.5	4.8 ± 0.9
Previous lactations	0.7 ± 0.3	1.5 ± 0.4	0.7 ± 0.3	1.8 ± 0.8
Days in milk at end of depletion period	95 ± 4	94 ± 6	94 ± 6	95 ± 5
Production of 4% FCM at end of depletion period	24.4 ± 3.5	25.0 ± 2.4	25.1 ± 2.2	30.3 ± 2.9
Live weight at end of depletion period	850 ± 93	928 ± 108	870 ± 68	965 ± 88
Days in gestation at end of experimental period	64 ± 18	74 ± 17	27 ± 24	54 ± 23

each set of four, individual cows were assigned according to a previously randomized allotment to one of two basal rations, 100% alfalfa, or 80% alfalfa plus 20% corn; and to one of two sequences of methods of substitution of corn for alfalfa, namely, estimated net energy (NE) and estimated total digestible nutrients (TDN), in the following sequences: NE-TDN-NE or TDN-NE-TDN, designated as Sequence A and Sequence B, respectively.

Cows on the 100% alfalfa ration were fed 3.0 lb. of dry matter of alfalfa per 100 lb. of live weight per day. Cows on the 80% alfalfa plus 20% corn ration were fed at such a rate that the ration equaled the 100% alfalfa ration in NE content. NE was calculated by the formula of Moore *et al. (22)*, derived

from Forbes' data, based on TDN values calculated from proximate analysis according to Schneider *et al. (26)*. During this feeding period, the depletion period, which continued through the 70th day, the ration allowance was adjusted for live weight on the 15th and 57th days.

On the 71st, 99th, and 127th days, the ration allowance of the cows on the 100% alfalfa basal was reduced to 95%, 90%, and 85%, respectively, of that fed during the 57th to 70th days. Cows on the 80% alfalfa plus 20% corn basal were fed accordingly equivalent amounts of NE. On the 85th day, 10 lb. of alfalfa was replaced with corn on either the NE or TDN basis, according to the assigned sequence. This was repeated on the 113th and 141st days. This feeding period followed a double change-over design *(6)* consisting of three 28-day periods. Each of these was made up of a 14-day standardizing period to establish lactation level, and a 14-day comparison period to test for differences in response to the two systems of substitution.

During the entire experiment, all cows received 0.3 lb. steamed bone meal daily, Guernseys and Jerseys 0.1 lb. iodized salt daily, and Holsteins 0.2 lb.

The alfalfa hay which was second-cutting, field-cured, and New York State grown, was chopped at a setting of ¾-inch. Samples of the hay, taken at 4-wk. intervals, graded on the average U.S. No. 2.2 alfalfa. They were composed of 5.1% foreign matter and the remainder of 93.0% alfalfa and 7.0% grasses and other legumes, and had 68.2% color and 28.5% leafiness. The corn, U.S. No. 1 dent, was fed coarsely ground. Average chemical composition of these feeds, as well as calculated NE and TDN content, are contained in Table 2.

Observation and analyses. All feeds, fed and refused, and milk yield were weighed to the nearest 0.1 lb. twice daily. Live weights were recorded to the nearest pound on the first, sixth and seventh day of each seven-day period.

Milk samples of the A.M. and P.M. milkings were taken every seven days during the depletion period, and daily during the successive standardizing and comparison periods, for fat determination by the Babcock method *(1)*. Four per cent fat corrected milk (FCM) was determined by the formula of Gaines *(12)*. Samples of feeds, fed and refused, were taken at each feeding and composited each seven-day period for proximate analysis *(1)*. TDN content of the weighbacks was computed from proximate analysis by the method of Schneider *et al. (25)* and NE as previously described.

The data were analyzed as outlined in the Statistical Appendix. In the analysis, the effect of advancing lactation upon the treatment responses was eliminated, based on each cow's linear decline. Only data for the last seven days of each standardizing and comparison period were used in the analysis. In one cow in the first replicate, missing values for the second standardizing and comparison period were derived, owing to a miscalculation of its feed allowance.

RESULTS

Average daily observed values of the feed intakes, the milk production criteria, and live weight changes for each standardizing and comparison period by

TABLE 2

Average proximate analysis, estimated total digestible nutrients, and net energy of feeds with their standard errors

Feed	Composition									
	Moisture	Crude protein	Crude fiber	Nitrogen-free extract	Ether extract	Ash	Ca [c]	P [c]	TDN	Therms NE
	(%)									
Alfalfa [a]	9.1 ±0.2	16.2 ±0.1	26.0 ±0.2	39.7 ±0.2	1.9 ±0.0	7.1 ±0.1	1.36	0.23	56.4 ±0.2	46.7 ±0.3
Corn [b]	11.6 ±0.2	8.6 ±0.0	1.7 ±0.0	72.8 ±0.4	4.0 ±0.0	1.3 ±0.0	0.02	0.28	79.2 ±0.2	80.9 ±0.2
Steamed bone meal [c]	2.65	.....	.....	.....	.....	.....	29.30	14.02	.....	.....

[a] Average of 94 samples.
[b] Average of 81 samples.
[c] Average of two samples.

experimental subgroupings are shown (Table 3). Responses to the substitution of corn for 10 lb. alfalfa, using either the TDN or NE system as affected by the previously fed basal ration, are given (Table 4). The values for average daily intake of therms of NE and pounds of TDN were based on each cow's actual feed intake and feed refusal and the proximate analyses for the particular week.

Feed intake. Basing the substitution of 10 lb. of alfalfa on the TDN system of evaluation resulted in the feeding of 7.0 lb. of corn; whereas, 5.7 lb. of corn were fed when the substitution was based on the NE system (Table 3). This resulted in an average daily advantage of 0.99 therm and 0.94 lb. TDN for the TDN substitution, when averaged over both basal rations (Table 4) and similar values for each basal ration.

It was anticipated that the NE and TDN intakes would remain constant when the substitution was based on the corresponding system. However, small differences were observed (Table 4) which most likely were due in part to variation in the weekly proximate analysis and in part to small feed refusals.

In the case of the 80% alfalfa plus 20% corn basal, substituting corn for alfalfa, especially in the last period, resulted in relatively low intakes of hay. However, no abnormally low butterfat tests were observed, even with intakes of 1 to 3 lb. per cow daily.

Milk production. When averaged over both basal rations, the advantage of basing the substitution of corn for alfalfa on the TDN system was a significant increase in production of milk, FCM, and fat, and conversely in significant decreases in the fat percentage (last row, Table 4). Such a response might have been anticipated from the previously indicated increases in energy intake. Examination of the lactation responses as affected by the basal ration fed yielded the following results: Substituting corn for alfalfa in the 100% alfalfa basal showed a very significant advantage for the TDN basis of substituting over the NE basis; whereas, no advantage was noted for the TDN basis when the substitution was made with the 80% alfalfa plus 20% corn basal. Even though the energy intake showed essentially the same advantage for both basals, 1.01 and 0.97 therms and 0.96 and 0.92 lb. TDN, respectively, for the TDN basis of substitution, the animals previously fed 100% alfalfa ration increased their production by 1.39 lb. FCM, while the animals fed the 80% alfalfa + 20% corn ration failed to respond. Of further interest was the observation that animals previously fed the 100% alfalfa ration responded to the substitution of corn on the NE basis, in spite of an intake of therms NE which remained essentially the same, by producing 0.94 lb. FCM more. In general, pounds of milk and fat showed the same trends as discussed for FCM, and the response for the fat percentage followed an opposite trend.

Live weight changes. The live weight data were analyzed in terms of average daily change in live weight during the last seven days of each standardizing and comparison period, rather than in terms of actual live weight. The use of actual live weights in change-over trials may possibly mask treatment differences, because of changes in digestive fill from period to period. In general, responses

TABLE 3

Average daily observed feed intakes, milk production, and live weight changes for each period

Criteria	Experimental groupings: Basal ration	Sequence of substitution[a]	Experimental period: I Std.	I Comp.	II Std.	II Comp.	III Std.	III Comp.
Alfalfa fed (*lb.*)	100% Alfalfa	A	26.9	16.9	25.4	15.4	24.1	14.1
		B	29.6	19.6	28.0	18.0	26.4	16.4
	80% Alfalfa + 20% corn	A	18.8	8.8	17.8	7.8	16.7	6.7
		B	21.0	11.0	19.5	9.5	18.8	8.8
Corn fed (*lb.*)	100% Alfalfa	A	0	5.7	0	7.0	0	5.7
		B	0	7.0	0	5.7	0	7.0
	80% Alfalfa + 20% corn	A	4.7	10.4	4.4	11.4	4.2	9.9
		B	5.2	12.2	4.8	10.5	4.7	11.7
Total NE intake (*therms*)	100% Alfalfa	A	12.6	12.5	11.7	12.6	11.3	11.2
		B	13.5	14.5	12.8	12.6	12.6	13.3
	80% Alfalfa + 20% corn	A	12.6	12.5	12.0	12.8	11.1	11.3
		B	13.8	15.0	12.9	12.8	12.4	13.4
Total TDN intake (*lb.*)	100% Alfalfa	A	15.2	14.1	14.2	14.0	13.6	12.5
		B	16.4	16.3	15.6	14.3	15.0	14.8
	80% Alfalfa + 20% corn	A	14.2	13.2	13.7	13.4	12.7	11.8
		B	15.8	15.9	14.8	13.6	14.2	14.0
Milk (*lb.*)	100% Alfalfa	A	23.3	24.1	20.8	22.8	18.0	19.2
		B	23.8	25.2	21.0	20.2	18.1	19.2
	80% Alfalfa + 20% corn	A	23.2	22.4	21.0	20.0	18.1	17.9
		B	27.5	26.3	24.6	23.3	22.1	21.5
Fat (%)	100% Alfalfa	A	4.5	4.3	4.5	4.3	4.6	4.5
		B	4.5	4.2	4.4	4.4	4.5	4.4
	80% Alfalfa + 20% corn	A	4.7	4.8	4.9	4.9	5.2	5.2
		B	4.8	4.7	4.7	4.8	4.9	5.0
Fat (*lb.*)	100% Alfalfa	A	1.0	1.0	0.9	0.9	0.8	0.8
		B	1.0	1.0	0.9	0.9	0.8	0.8
	80% Alfalfa + 20% corn	A	1.0	1.0	1.0	0.9	0.9	0.9
		B	1.3	1.2	1.1	1.1	1.0	1.0
4% FCM (*lb.*)	100% Alfalfa	A	24.4	24.4	21.6	23.2	20.0	20.1
		B	25.0	25.6	22.0	21.0	19.1	20.1
	80% Alfalfa + 20% corn	A	25.1	24.4	23.0	22.1	20.7	20.4
		B	30.3	28.6	26.6	25.3	24.7	24.1
Change in live weight (*lb.*)	100% Alfalfa	A	+0.1	+0.7	+1.0	0.0	+0.2	+0.2
		B	+0.2	+0.5	+1.2	+0.2	−1.3	+0.7
	80% Alfalfa + 20% corn	A	0.0	−1.8	+0.7	−1.8	+0.4	+1.1
		B	−0.7	−1.2	−0.5	+0.4	+1.5	−0.1

[a] Sequence A—NE-TDN-NE; Sequence B—TDN-NE-TDN.

in changes of live weight to the substitution of corn for alfalfa were not significant. However, of interest is the observation that there was no advantage for substituting on the basis of TDN in the case of the 100% alfalfa basal, 0.01 lb., while there was a pronounced loss of live weight for the 80% alfalfa plus 20% corn basal, −1.40 lb., which is the same trend as observed for the response of FCM.

Two factors which may have influenced the results of this study are that protein intake, either as per cent of ration or total daily intake, was not equalized between basal rations or methods of substitutions, and that the proportion of roughage to concentrate was not held constant within basals upon substitution. Unfortunately, these effects, if real, were not measurable in this study.

Calculated NE values of alfalfa. To explore whether a difference existed in the utilization of the alfalfa fed in this study, between the 100% alfalfa basal group and 80% alfalfa plus 20% corn basal, NE values were computed for the alfalfa for each basal. Only data from the three standardizing periods, when the basals were fed on an equivalent NE intake per unit of live weight, were utilized. In these calculations, 6.0 therms per 1,000 lb. live weight to the 0.7 power were allowed for maintenance and 4% FCM at 0.30 therm per pound. The NE value of corn was based on the particular week's proximate analysis and derived as previously described. Based on the individual cow's requirement for maintenance and production, the net energy value of the alfalfa was found to be 45.53 therms per 100 lb. when fed alone, as in the 100% alfalfa basal, and 49.92 therms per 100 lb. when fed in conjunction with corn, as in the 80% alfalfa plus 20% corn basal. Although this is not very conclusive evidence, it indicated that the nutritive value of the alfalfa may have been increased by the addition of limited amounts of corn.

DISCUSSION

This study substantiated essentially the results of Huffman *et al. (14)* and other workers *(9, 27)* that, when part of an all-hay ration is substituted with a concentrate on an equivalent TDN basis, milk production will increase. That such an increase appeared owing to increased intake of NE, as pointed out by Saarinen *(24)* and Irvin *(15)*, was also substantiated. However, in this study not all the increase appeared to be due to the increased NE intake, inasmuch as the cows fed 100% alfalfa previously showed increased milk production when 10 lb. of alfalfa was replaced with equivalent amounts of NE from corn, which is in agreement with the study of Loosli *et al. (19)*. The above observation, and the fact that cows fed the 80% alfalfa plus 20% corn ration failed to respond to the increased energy, when corn was substituted on the TDN rather than the NE basis, suggested the possibility that the effective energy content of a ration may to some degree be affected by the proportion of roughage to concentrate it contained, or by other factors, such as protein intake and so on.

The TDN basis of estimation of the nutritive value of feedstuffs has long been suspected to change with the proportion of roughage to concentrates: Beach *(3)* in 1906 showed that 4.5 lb. of TDN from corn was equivalent to 7.1 lb. of TDN from hay for the maintenance of steers, when both feedstuffs were fed by

TABLE 4

Responses to the substitution of corn for alfalfa of feed intakes, milk production, and live weight changes

Response to substituting corn for alfalfa	NE intake	TDN intake	Milk	Fat	Fat	FCM	Live weight
	(therms/day)	*(lb/day)*	*(lb/day)*	*(%)*	*(lb/day)*	*(lb/day)*	*(lb/day)*
100% alfalfa basal							
TDN basis	1.16 (0.27)[a]	0.19 (0.24)	3.11 (0.81)	−0.24 (−0.12)	0.083 (0.034)	2.33 (0.74)	0.28 (1.30)
NE basis	0.15 (0.27)	−0.77 (−0.24)	1.55 (0.81)	−0.13 (−0.12)	0.032 (0.034)	0.94 (0.74)	0.27 (1.30)
Advantage for TDN over NE basis	1.01 (0.30)	0.96 (0.29)	1.56 (0.71)	−0.11 (−0.14)	0.051 (0.034)	1.39 (0.77)	0.01 (1.56)
80% alfalfa basal							
TDN basis	1.31 (0.27)	0.25 (0.24)	0.42 (0.81)	−0.12 (−0.12)	−0.003 (−0.034)	0.10 (0.74)	−1.98 (−1.30)
NE basis	0.34 (0.27)	−0.67 (−0.24)	0.22 (0.81)	−0.04 (−0.12)	−0.002 (−0.034)	0.03 (0.74)	−0.58 (−1.30)
Advantage for TDN over NE basis	0.97 (0.30)	0.92 (0.29)	0.20 (0.71)	−0.08 (−0.14)	−0.001 (−0.034)	0.07 (0.77)	−1.40 (−1.56)
Average of both basals							
TDN basis	1.23 (0.19)	0.22 (0.17)	1.77 (0.58)	−0.18 (−0.08)	0.040 (0.024)	1.22 (0.52)	−0.85 (−0.92)
NE basis	0.24 (0.19)	−0.72 (−0.17)	0.88 (0.58)	−0.08 (−0.08)	0.015 (0.024)	0.49 (0.52)	−0.16 (−0.92)
Advantage for TDN over NE basis	0.99 (0.21)	0.94 (0.20)	0.89 (0.50)	−0.10 (−0.10)	0.025 (0.024)	0.73 (0.54)	−0.69 (−1.10)

[a] Response needed for significance at $P = 0.05$.

themselves. Also, the NE basis of estimation may not be additive as the proportion of hay to corn is changed. Evidence for this is provided by Forbes *(11)*, who found that the NE value of corn when fed alone was less than when corn and alfalfa hay were fed together. Indirect evidence in feeding experiments with lambs, calves, and steers indicates there is possibly an optimum proportion of roughage to concentrates for maximum growth and gross feed efficiency *(8, 10, 16)*. It would, therefore, appear that such might be the case for lactation response in dairy cows, as is suggested by the data of Martin *et al. (21)*, even though the curvilinearity in their data was not statistically significant.

Considerable controversy currently exists as to the most applicable predictor of the nutritive value of various feedstuffs for dairy cattle *(4, 7, 17, 22, 23, 28)*. These studies and reviews have involved not only the use of TDN and NE but also other systems such as starch equivalent, productive energy, metabolizable energy, and digestible energy. Most of these have been concerned with comparisons among systems or establishment of nutritive values over very limited ranges of combinations of roughage and concentrates. The aforementioned studies (as well as the one reported herein on the substitution of corn for alfalfa) cover only a limited range of possible combinations of roughages and concentrates, and at only a very narrow range of nutritive intakes. Therefore, it would appear that additional data need to be collected, with lactating dairy cows, at several fixed planes of nutrition, and at each plane of nutrition different combinations of hay to grain covering the possible range from all roughage to all grain. A recent study by Bloom *et al. (5)* has provided some preliminary information of this type. Such data possibly would allow present systems of feed evaluation to be used, but modified with a factor(s) descriptive of the type of ration fed and the plane of nutrition. The establishment of a good possible functional relationship of milk production to type of ration and plane of nutrition awaits collection of appropriate data *(2, 13)*. Such data would also lend themselves to economic interpretation of optimum input-output price ratios and rates of substitution of feedstuffs.

Accurately to evaluate feed utilization by the lactating dairy cow, both milk production and body composition changes need to be evaluated simultaneously *(18)*. Because methods to estimate changes in body composition and the contribution of rumen fill to live weight are now in the developmental stage, most studies including the one reported here are as yet unsuitable to evaluate successfully and simultaneously the two measures of energy utilization, milk production and body composition.

ACKNOWLEDGMENTS

The authors are indebted to B. A. Donohue and T. Watts for the care of the experimental animals and help in the collection of the data, and to H. J. Fisher, Connecticut Agricultural Experiment Station, New Haven, for the proximate analyses of the feedstuffs. Helpful suggestions by R. H. Benson, R. F. Davis, I. F. Fellows, G. C. Judge, C. F. Huffman, J. K. Loosli, and J. T. Reid aided the authors in the analyses of the data and preparation of the manuscript, for which we are most grateful.

STATISTICAL APPENDIX

The Analysis Problem

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The experimental design of the study reported here may be represented as follows:

Period	Treatment sequence			
	1	2	3	4
1	a	a	a'	a'
2	b	c	b'	c'
3	a	a	a'	a'
4	c	b	c'	b'
5	a	a	a'	a'
6	b	c	b'	c'

In the table:

a, a' represent the two basal rations used, i.e., 100% alfalfa, and 80% alfalfa + 20% corn, respectively.

b, b' represent the substitution of corn for alfalfa in the basal rations on the NE basis.

c, c' represent the substitution of corn for alfalfa in the basal rations on the TDN basis.

The basic design was replicated six times.

The comparisons b *vs.* c and b' *vs.* c' presented no problem in analysis, because the observations on a and a' could be neglected and the data analyzed, as for switch-back trials by the method of Brandt *(6)*. For the comparisons of a with b and c, and of a' with b' and c', however, a method of analysis had to be formulated. The principal problem was to make the latter comparisons fair.

A straight-out comparison of corn and alfalfa by the use of simple averages could be biased, because a and a' did not appear in the same experimental periods as did b, b', c, or c'. One factor which, under the treatment arrangement used, could favor a and a' is the decline in production as lactation advances. Correction for this was made in the analysis by assuming a linear decline different for each cow. Another factor which could have caused bias, if all replications of the basic design had been started at the same time, is the shift in general environment from one period to another. Because the several replications and usually the several cows in a replication were started at different times, however, it was assumed that the treatments favored changed from cow to cow in an essentially random fashion and that no bias was introduced. Examination of the data indicated that this assumption was fairly safe to make in the present experiment. In general, however, the assumption of no period effects is not a safe one, and a design not requiring such an assumption is preferable *(20)*.

Comparison of alfalfa with corn.

The first step was to compute the quantity

$$P = 5y_1 + 11y_2 - 8y_3 + 8y_4 - 11y_5 + 5y_6$$

for each cow, where $y_1, y_2, \ldots, y_6$ are the performances during periods 1,2, . . . , 6, respectively. For the cow with the missing data, P was taken as the average of the P's for the other cows on the same sequence. It is seen that P is clear of the linear time trend, because

$$-5(1) + 11(2) - 8(3) + 8(4) - 11(5) + 5(6) = 0.$$

In order to outline the analysis, let

$P_{ij} = P$ value for the jth cow on the ith sequence ($i = 1,2, \ldots, 4$; $j = 1,2, \ldots, n$; here $n = 6$)

$G_i =$ sum of the P values on the ith sequence.

The error mean square on the per-observation basis was computed as

$$s^2 = \frac{1}{420(4)(n-1)} \left[\sum_i \sum_j P_{ij}^2 - \sum_i \frac{G_i^2}{n} \right]$$

in which $4(n-1) =$ degrees of freedom for error. Here d.f. $= 4(6-1) = 20$, basically, but because of the missing value for one cow, d.f. $= 19$, actually.

The advantage for corn substitution averaged over both basal rations *(D)* and its standard error *(s_D)* are given by

$$\frac{G_1 + G_2 + G_3 + G_4}{96n} \text{ and } \sqrt{\frac{35s_P^2}{192n}}$$

respectively. The advantages for corn in the 100% alfalfa ration and in the 80% alfalfa ration are given by

$$d(100) = \frac{G_1 + G_2}{48n} \text{ and } d(80) = \frac{G_3 + G_4}{48n}$$

both with standard error, $s_D\sqrt{2}$. The additional advantage that corn substitution in the 100% alfalfa ration has over corn substitution in the 80% alfalfa ration is

$$d(100-80) = \frac{G_1 + G_2 - G_3 - G_4}{48n}$$

with standard error $2s_D$.

Comparison of TDN substitution with NE substitution:

Following Brandt (1938), the quantity

$$Q = y_2 - 2y_4 + y_6$$

was computed for each cow. For the cow with missing data, Q was taken as the average of the Q's for the other cows on the same sequence. Q is orthogonal to the P previously computed. It is also clear of linear time trends, since

$$1(2) - 2(4) + 1(6) = 0.$$

To describe the computations, let

$Q_{ij} = Q$ value for the jth cow on the ith sequence
$H_i =$ sum of the Q's on the ith sequence

The error mean square is given by

$$s_Q^2 = \frac{1}{6(4)(n-1)}\left[\sum_i \sum_j Q_{ij}^2 - \sum_i \frac{H_i^2}{n}\right]$$

Here again the d.f., $4(n-1)$, were reduced by one, because of the missing value.

The advantage for TDN substitution was compared to NE substitution averaged over both basal rations (E) and its standard error (s_E) are

$$\frac{-H_1 + H_2 - H_3 + H_4}{8n} \text{ and } \sqrt{\frac{3s_Q^2}{8n}},$$

respectively. The advantages in the 100% alfalfa ration and in the 80% alfalfa ration are

$$c(100) = \frac{-H_1 + H_2}{4n} \text{ and } c(80) = \frac{-H_3 + H_4}{4n}$$

both with standard error $s_E\sqrt{2}$. The additional advantage that TDN substitution in the 100% alfalfa ration has over TDN substitution in the 80% alfalfa ration is

$$d(100-80) = \frac{-H_1 + H_2 + H_3 - H_4}{4n}$$

with standard error $2s_E$.

Comparison of alfalfa with corn substituted on either the TDN or the NE basis.

When corn is substituted on the TDN basis, the advantage over the basal rations are

$$D + \frac{1}{2}E, d(100) + \frac{1}{2}c(100) \text{ and } d(80) + \frac{1}{2}c(80),$$

respectively, for the two basals combined, for the 100% alfalfa ration and for the 80% alfalfa ration. Similarly, when corn is substituted on the NE basis the advantages over the basal rations are

$$D - \frac{1}{2}E, d(100) - \frac{1}{2}c(100), \text{ and } d\ (80) - \frac{1}{2}c(80)$$

The contrasts that involve both basals have a standard error

$$s = \sqrt{\frac{35s_P^2 + 18s_Q^2}{192n}}$$

and those involving a single basal have a standard error $s\sqrt{2}$.

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RATE OF ABSORPTION OF CAROTENE AND OF VITAMIN A FROM THE ALIMENTARY TRACT OF DAIRY CALVES.[1] II. EFFECTS OF METHODS OF DISPERSION AND OF ADMINISTRATION

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SUMMARY

Different dispersions and methods of administration of vitamin A–active supplements on their apparent absorption by calves were studied. Vitamin A in an oily menstruum, an homogenized emulsion, and an aqueous dispersion—and carotenoids—in an oily solution and an emulsion—were mixed in a filled milk and fed via nipple. Vitamin A supplements, as an oily medium and as an aqueous dispersion, also in milk, were administered by a stomach tube. The rate of administration was 1,000 I.U./lb body weight. Changes in concentrations of vitamin A and of carotenoids in blood plasma, collected at 3-hr. intervals during a 15-hr. period after feeding the supplements, were the criteria of absorption. Concentrations of vitamin A in blood plasma increased and subsequently decreased more rapidly than did those of carotenoids. Emulsifying oily supplements in milk by homogenization significantly enhanced the apparent absorption of carotenoids but did not improve markedly the absorption of vitamin A. Aqueous dispersion of vitamin A effected more rapid passage into the blood and a higher maximum level than did either the emulsion or the oily solution. Ingestion of vitamin A by nipple resulted in more rapid absorption and higher concentrations in the plasma than did administration by stomach tube.

The frequent occurrence of vitamin A deficiency in cattle is the primary basis for continued exploration of factors affecting efficiency of utilization of this vitamin and its precursor. Among the many conditions that may influence the physiological economy of utilization are forms of dispersion *(4, 12)* and methods of administration *(7, 8)*.

The advent of dispersing agents has stimulated investigations of the effects of these preparations on the absorption of vitamin A and of carotenoids. Extensive work with simple-stomached animals has revealed that emulsification and aqueous dispersions enhance absorption of vitamin A–active compounds *(4, 12)*. Results from similar studies with cattle, though few, generally are concordant with observations on other species *(3, 7, 8, 9)*.

In view of the unique anatomical and functional characteristics of the ruminant stomach, the rate of passage of substances along the alimentary tract and the extent of exposure to biochemical agents may be affected by the physical state of the material and the methods of administration. Apparently, when vitamin A–active media pass directly into the abomasum, the rate of absorption is faster and the concentrations in the blood are greater than when the supplements enter the rumeno-reticular cavity *(7, 8)*.

Received for publication July 18, 1957.

[1] Journal Paper No. J-3226 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 814.

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The need for further elucidation of the apparent absorption responses of calves to various forms of dispersions of vitamin A and of carotenoids, and to different methods of administering them, constituted the bases for the study presented herein.

EXPERIMENTAL PROCEDURE

Design. The principal variables in the investigation were, *(a)* different vitamin A–active supplements incorporated in a filled-milk diet, *(b)* methods of dispersing these supplements in the milk, and *(c)* the manner of administering vitamin A–supplemented milk to calves. Effects of these treatments on apparent absorption of carotenoids and of vitamin A were investigated in two primary series of trials, I and II, and in a secondary trial, which was a check test. In Series I and in the check tests, 12 calves of the Holstein breed were used, and in Series II, eight calves from Series I were continued. Each animal used in the respective series of trials was subjected to the treatments indicated (Table 1). The basic design employed in determining the sequential order of treatments within each series of trials was a Latin square.

TABLE 1

Methods of dispersing various concentrates of vitamin A and of carotenoids in filled milk and procedures in administering the supplemented milk to experimental calves

Series of trials	Vitamin A–active supplements			Method of incorporating supplements in milk	Mode of administering milk to calves[f]
	Substance	Medium	Potency[e] *(IU/g)*		
I	Vitamin A[a]	Fish liver oil	30,000	Homogenization Hand-stirring	Nipple feeder Nipple feeder
	Carotenoid[b]	Cottonseed oil	50,000	Homogenization Hand-stirring	Nipple feeder Nipple feeder
Basal-diet check		No supplement			Nipple feeder
II	Vitamin A[c]	Oil concentrate	100,000	Hand-stirring	Nipple feeder Stomach tube
		Aqueous dispersion[d]	12,500	Hand-stirring	Nipple feeder Stomach tube

[a] Supplied by the Borden Co., New York, through the courtesy of L. T. Wilson.
[b] Primarily β-carotene, purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.
[c] Supplied by Distillation Products, Inc., Rochester, N. Y., through the courtesy of P. L. Harris.
[d] Distilled natural ester concentrate emulsified by Tween 80 (polyoxyethylene sorbitan monoöleate) in the ratio of seven parts of dispersing agent to one part of oil.
[e] Listed values.
[f] Standard rate of administration was 1,000 I.U/lb of body wt.

Test diets. The basal diet, to which the respective vitamin A–active supplements (Table 1) were added, was a filled milk containing 10% nonfat milk solids, 3% hydrogenated soybean oil, and 87% water. The oil and the milk solids were dispersed in warm water, approximately 38° C., by thorough mixing followed immediately by homogenizing at 3,000 p.s.i. The respective vitamin A and carotenoid carriers (Table 1) were incorporated in the filled milk either *(a)* by adding the supplement during the mixing of the basal ingredients and

subsequently dispersing in the homogenization process, or *(b)* by adding it after homogenization and later dispersing by hand-stirring. To maintain a uniform distribution of this unhomogenized supplement in the milk, the mixture was agitated continuously as it was administered to the calf.

Feeding and management. During the three days immediately following the birth of each calf, it consumed colostrum directly from its dam. Thereafter, except when a test diet was given, fresh whole milk was fed routinely from a nipple pail. The rate of feeding was 10 lb. milk per cwt. of calf per day. In addition to the whole milk, each calf received daily a maximum of 0.5 lb. of a simple concentrate mixture fortified with minerals and vitamin D.

When the various treatments outlined (Table 1) were imposed on the calves, a regular morning feeding of whole milk was replaced with an equal quantity of one of the test diets (filled milk plus one of the supplements studied). No other feed was consumed during the subsequent 15 hr.

The test diets were administered, as previously described *(8)*, either from a nipple-feeder or by a stomach tube. Presumably, when the former method was used, nearly all the milk passed directly into the abomasum *(6, 15)*; whereas, when the latter was employed, passage was directly into the rumeno-reticular cavity *(8)*. Thus, to avoid flooding of these fore compartments of the stomach and consequent spillage into the abomasum, the quantity of filled milk used as a vehicle for the supplements (Table 1) was only one-third to one-half the total feed allowance when the stomach-tube administration was used. The remainder of the filled-milk allotment was given via a nipple feeder.

Trials of Series I were initiated when the calves were two weeks of age and were repeated at weekly intervals thereafter, until each calf had been subjected to treatments listed for this series (Table 1); whereas, trials of Series II were initiated when the calves were about 8 wk. of age and were continued through the treatment sequence at biweekly intervals. The special check trial, designed to ascertain responses to the basal diet per se, was conducted during the interim between Series I and II.

Sampling and analyzing blood. The criteria of responses to the treatments listed (Table 1) were carotenoid and vitamin A patterns of absorption, as reflected in diurnal changes in concentrations of these constituents in blood plasma of the calves. Blood samples were collected from the jugular vein of the calves immediately preceding the administration of the test diets and at 3-hr. intervals thereafter until the 15th hr. During the initial stages of this investigation, concentrations of carotenoids and of vitamin A in the samples of blood plasma were determined by a procedure described by Squibb *et al. (14)*; subsequently, however, the method was modified to include saponification *(1)*.

RESULTS

Series I. *Effect of dispersion procedure on concentrations of vitamin A and of carotenoids in blood plasma.* Diurnal patterns resulting from administrations of the respective vitamin A–active supplements, dispersed by hand-stirring

and by homogenization, are shown (in the first two sections of Figure 1). Mean values of vitamin A and of carotenoids in the samples of plasma from blood collected immediately prior to administrations of test diets were used as the reference base level, 0. The curves, therefore, represent changes from this pre-treatment value.

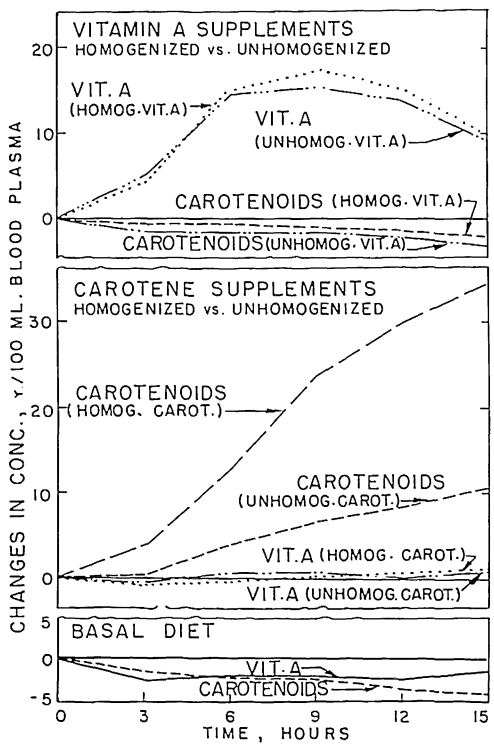


FIG. 1. Comparative diurnal changes in mean concentrations of vitamin A and of carotenoids in the blood plasma of calves receiving oily spplements, dispersed in a basal filled milk by homogenization and by hand-stirring.

The manner of dispersing the vitamin A supplement in the diet effected no pronounced difference in apparent absorption patterns in calves (as is revealed in the top section of Figure 1). Concentrations of plasma vitamin A increased to a peak between the 6th and the 12th hr., and declined subsequently. Levels of carotenoids in the same series of samples of blood plasma were not affected significantly by the form of vitamin A dispersion. Trends, however, during the 15-hr. period, were slightly downward.

In contrast to the vitamin-A curves, response patterns (middle section, Figure 1) resulting from feeding of carotenoids dispersed by the two methods were markedly different. Within the period of observation, the rates of increases and the magnitude of concentrations of carotenoids in blood plasma were greater from the homogenized supplement than from that which was hand-mixed. An analysis of variance of linear components, which were from a combination

of all values obtained from the sequential set of six samples collected during the 15-hr. period, indicated a significant difference ($P < 0.01$) in patterns of the carotenoid curves. Increases in plasma vitamin A concentrations, corresponding to the elevation in carotenoids, were only slight and were similar for the two forms of dispersions.

A comparison of the diurnal curves for vitamin A and for carotenoids indicates that the rate of increase to a crest was more rapid for the former than for the latter.

Effect of the basal diet on concentrations of vitamin A and of carotenoids in blood plasma. During the 15 hr. after the unsupplemented basal filled milk was fed, mean levels of carotenoids and of vitamin A in blood plasma of the calves decreased 3.7 γ/100 ml. and 1.6 γ/100 ml., respectively (bottom section, Figure 1). These reductions, evaluated by analysis of variance, were significant at the $P < 0.01$ level. Because values for samples of plasma collected immediately prior to administrations of test diets were used as reference bases in trials of Series I, the real magnitude of increases effected by supplements probably was greater than apparent values indicated.

Series II. *Effect of form of dispersion of vitamin A and of method of administration on concentrations of vitamin A and of carotenoids in blood plasma.* In Figure 2, curves showing means of observed concentrations, instead of differences from pretreatment values, as presented in Series I, indicate that the aqueous dispersion effected a more rapid increase of vitamin A in blood plasma and a higher concentration than did the oily medium. Intake via nipple also

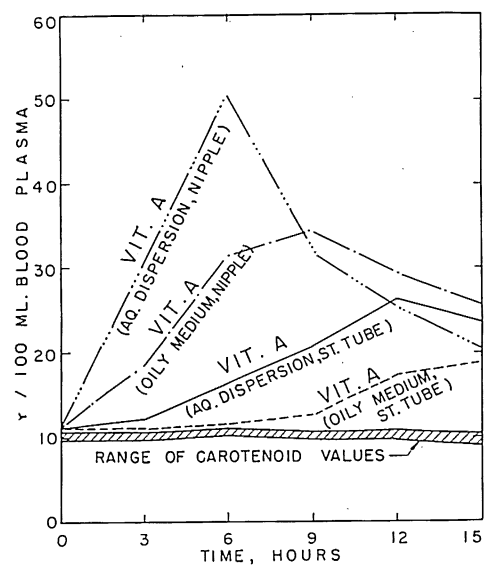


FIG. 2. Diurnal changes in the mean concentrations of vitamin A and of carotenoids in the blood plasma of calves that received vitamin A supplements, an aqueous dispersion and an oily mentruum, mixed in a basal filled milk, and administered by a nipple feeder and by a stomach tube, respectively.

resulted in a more rapid rate of increase and a higher level, within the 15-hr. period, than did administration by stomach tube. Patterns of response from administration of the two kinds of vitamin A supplements (oil concentrate and aqueous dispersion) by the two different methods (nipple feeder and stomach tube) indicate that rates of increases and maxima of concentrations are related positively. Furthermore, curves cresting within the 15-hr. period indicate that the higher the maximum level the more precipitous was the subsequent decline. The duration of observation, however, was too brief to reveal the general pattern resulting from giving the oily menstruum by stomach tube. The crest for the aqueous dispersion given from a nipple feeder occurred between 3 and 9 hr., which increase was more rapid than noted for vitamin A in oil administered similarly.

The maxima of the plasma vitamin A increases among the calves covered a wide range in the sequence of trials in Series II, but the relative order of response by individuals, as each was changed from one treatment to another, was strikingly uniform. The data revealed no apparent effects of either carry-over or age on responses.

Concentrations of carotenoids in the blood plasma were remarkably consistent throughout this series of trials. The spectrum of values (Figure 2) for the 15-hr. period apparently was not markedly affected by the vitamin A absorbed, even though the terminal level was slightly lower than the initial.

DISCUSSION

Results from the series of trials with calves, in accordance with findings reported for other species *(4, 12)*, indicate, for the most part, a close relationship between the forms of dispersions of carotenoids and of vitamin A in the diet, and the diurnal changes in concentrations of these constituents in blood plasma. Though it is recognized that the blood picture does not necessarily reflect accurately the efficiency of absorption *(7, 12)*, the response patterns are regarded as an acceptable index *(12)* in most instances.

The finding that emulsification of the carotenoid supplement by homogenization markedly enhanced the passage of this substance into the blood stream of calves *(16)* has been substantiated *(3, 9)*. Kon *et al. (9)* also found that when calves received carotene in an emulsion (homogenized), the resulting concentrations in the blood were as high as those from the consumption of an aqueous dispersion of carotene, and were maintained at the high level longer. Thus, state of dispersion of the carotenoid vehicle evidently plays an important role in absorption responses by calves as well as by other species.

Even though emulsification may not be so essential for absorption of vitamin A as it is for the epithelial passage of carotenoids *(4)*, the failure of the homogenized vitamin A concentrate to augment the elevation of vitamin A in plasma of calves significantly above the level resulting from the consumption of an oily solution, was contrary to expectations and discordant with findings from investigations with rats *(10)*. If the interpretation of the role of particle size in intesti-

nal absorption *(5)* is applicable to the utilization of vitamin A, the foregoing apparent discrepancy may be attributed to the failure of the homogenization pressure of 3,000 p.s.i. to reduce the particle size of the oily vitamin A vehicle sufficiently to be reflected prominently in the vitamin A concentrations in the blood. Further investigation, however, is necessary to explain decisively the results obtained with calves.

Differences in the diurnal patterns of vitamin A and of carotenoids, the former increasing to maxima more rapidly than the latter, and subsequently decreasing, are in accord with previous observations *(8)*. Vitamin A apparently not only is absorbed from the alimentary tract more readily than are carotenoids *(4)*, but also is removed from circulation more rapidly *(9)*.

The effectiveness of aqueous dispersions in accelerating the rate of transition of vitamin A to the blood of calves and in raising the peak of concentration is in harmony with observations on other species *(4, 12)*. The findings with calves *(16)* have been substantiated by Kon *et al. (9)* and supported by Jacobson *et al. (7)*. Sobel *et al. (13)* also found that an oily vitamin A concentrate dispersed in a sodium chloride solution by sorethytan laurate, and administered by stomach tube to lactating cows, effected a slightly higher concentration of vitamin A in the blood and a many-fold higher level in milk than did giving the same amount of vitamin A dissolved in corn oil.

Response patterns from different methods of administration (Series II) substantiate previous findings *(8)*, in which vitamin A supplements fed by nipple effected a more rapid increase in the concentrations of vitamin A in blood plasma and apparently higher maxima than did similar supplements administered by stomach tube. Among the many factors that may be involved, the most probable *(8)* included differences in pregastric exposure and in rate of alimentary passage, thereby possibly effecting a difference in losses of vitamin A potency and in efficiency of absorption. Obviously, explorations of a more definitive type are necessary to resolve the physiological complexity relating to the foregoing responses.

Unfortunately, the 15-hr. sampling period employed was too brief to characterize the entire pattern of changes resulting from the two methods of administration. Previous observations *(8)*, however, indicate that the durations of apparent absorption, pre- and post-maxima, were longer from the administration by the stomach tube than by the nipple feeder. Thus, in the total utilization of vitamin A initially deposited in the rumeno-reticular cavity, the efficiency from slow passage and uptake may be greater than the diurnal picture would indicate *(7)*.

The slight depression of levels of carotenoids in blood plasma following the absorption of vitamin A was not any greater than the decreases noted when the basal diet was fed. Also, the minor increase of vitamin A following uptake of carotenoids was statistically insignificant. Though the changes were in the direction anticipated, they were less than expected. These responses might have been amplified if the base levels of carotenoids in the blood had been higher when

vitamin A was administered, and if the vitamin A had been lower when the carotenoids were given. Kon *et al. (9)* found that within 4 hr. after the administration of carotene to calves low in vitamin A, increases in both alcohol and ester forms of this vitamin occurred. This finding is not in accord with that reported by Crowley and Allen *(3)*. Factors affecting interrelationships of vitamin A and carotenoids in the blood of calves receiving massive doses of either of these substances remain somewhat obscure.

Inasmuch as the observed levels of vitamin A and of carotenoids in the blood plasma represent only the state at the time of sampling, interpretations of the physiological significance of the response patterns in terms of net utilization are challengeable. However, recent investigations by Jacobson *et al. (7)* showed that when vitamin A in an aqueous dispersion was given to calves, not only absorption but also storage was greater than when the same unitage of vitamin A was administered in an oily solution. Since there is some doubt about the ability of cattle to convert injected carotenoids to vitamin A *(2, 9)*, the nutritional advantage from accelerating absorption of the precursor may not be so great as the gains from rapid passage of vitamin A. Kon *et al. (9)* found that when aqueous dispersions of carotene were given orally to calves ". . . carotene and vitamin A appeared in the blood more rapidly and in greater concentration, and much more carotene was deposited in the liver . . ." than when carotene in oily solution was administered, though the vitamin A stores were the same.

The findings reported herein indicate the potential importance of states of dispersions and of methods of administration of preparations of vitamin A and of carotenoids in the physiological economy of their utilization. These observations have possible therapeutic as well as prophylactic applications in calf husbandry. Utilization failures associated with impairment of absorption of carotene *(9)* or of vitamin A *(11)* in diarrhetic calves perhaps may be overcome partially through the administration of large amounts of aqueous dispersions of vitamin A. Acceptance of vitamin A–active aqueous dispersions for use in a normal calf regimen depends not only on improved efficiency of absorption but also on the cost of the preparations and their stability in storage.

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AN EVALUATION OF THE CHROMIC OXIDE METHOD FOR DETERMINING DIGESTIBILITY

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SUMMARY

Eight lactating dairy cows were used in a ten-day digestion trial, in which the conventional total collection method of determining digestibility was compared with the grab-sampling technique using chromic oxide as an indicator. Variations in the excretion of chromic oxide and crude fiber at 2-hr. intervals over a 24-hr. period were studied. Statistical analysis showed no significant differences between the two methods of determining the digestibility of the rations. Analyses of samples from the total fecal composites for the first six days of the ten-day trial revealed coefficients of digestibility comparable to those obtained for the entire period. Considerable variations were found in the chromic oxide content of the feces samples taken at various hours of the day, regardless of whether chromic oxide was administered once or twice daily. These variations make it difficult to select a sampling period which will give approximately 100% recovery for all cows for a given day; however, these data indicated this can be overcome in digestion studies by sampling the feces for a ten-day period. The hourly variations in the excretion of chromic oxide were not associated with the excretion of crude fiber.

Nutritionists are still confronted with the problem of measuring the feeding value of various rations and forages. For the past few years, considerable attention has been given to indicator methods, for determining the digestibility and intake of forages. Compared to the conventional methods for determining digestibility, indicator methods show great advantages, in saving time, labor, and equipment.

Hamilton *et al. (5)* and Crampton *et al. (3),* using sheep, found the estimated coefficients of digestibility, using chromic oxide, to be comparable to those obtained from total collections. The former workers found that the length of the sampling period should be three or more days in length, in order to reduce sampling errors. The latter workers reported data which would indicate that the type of ration fed influences the results obtained from the chromic oxide method. When hay alone was fed, poor results were obtained, and upon the addition of grain to the ration the chromic oxide method checked more closely with the conventional method. This difference was explained on the basis of recovery of the chromic oxide, which was found to be only from 85 to 87% three days after chromic oxide administration had stopped, for sheep receiving the hay ration, as compared to from 98 to 99% for the same animals receiving a hay plus grain ration. This finding would indicate that the crude fiber level in the ration might conceivably affect the excretion of chromic oxide.

Kane *et al. (8, 9)* obtained good results in estimating the digestibility of alfalfa hay and silage fed to dairy cows, using the lignin ratio and chromic

Received for publication August 9, 1957.

oxide method, when the fecal samples were removed from the total fecal collection. However, these results could be misleading, since sampling errors for both chromic oxide and the various nutrients would be eliminated. No advantage would be gained by using the chromic oxide method if total collections of feces had to be made in order to obtain a representative sample with respect to chromic oxide and the different nutrients. From a practical standpoint, the accuracy of the chromic oxide method can be determined only by grab sampling at hours of the day when the chromic oxide content of the feces approximates 100% recovery.

Some workers have been hesitant about accepting the chromic oxide method, because of the unknown cause of diurnal variations in its excretion, and the inability quantitatively to recover the amount ingested. Kane *et al. (10)* found highly significant differences between the levels of chromic oxide in A.M. and P.M. fecal samples. The highest concentration of chromic oxide in the feces was found near 9 A.M., followed by a gradual decline until 9 P.M. These variations in the excretion of chromic oxide were attributed to unknown internal biological laws.

Hardison *et al. (6)*, working with steers, and Lancaster *et al. (11)* and Smith *et al. (13)* with cows, found wide variations in the excretion of chromic oxide when the animals were grazing. Hardison *et al.* reported that steers hand-fed pasture forage exhibited an excretion pattern of chromic oxide similar to barn-fed cows *(6)* but different from grazing animals. This difference in excretion curves of grazing and hand-fed animals was attributed to time of feed intake. Smith found that the number of times (once or twice daily) or the mode of administration (capsule or in the feed) of chromic oxide had little or no effect upon the degree of accuracy of estimating fecal output of cows on pasture. Recently, Kameoka *et al. (7)* reported from a study with sheep that the kind of feed or the time of feed ingestion had no effect upon the diurnal variations in the excretion of chromic oxide. It was concluded that the daily variation in chromic oxide excretion was a normal occurrence amounting to about 10%.

The objectives of the present study were: *(a)* to compare the chromic oxide method with total collections of feces for determining digestibility; *(b)* to compare a six-day total collection period with a ten-day period; *(c)* to study the effects of the number of administrations of chromic oxide daily upon the hourly variations of this inert material; and *(d)* to check the possible relationship of the hourly excretion of chromic oxide to crude fiber.

EXPERIMENTAL PROCEDURE

I. Digestion studies. Eight lactating dairy cows, receiving four different rations, were selected on the basis of dry matter intake, milk production, and body weight. The grouping of the cows, along with the four different rations fed, is given (Table 1). During a ten-day preliminary period, the cows were given 5 g. of chromic oxide in a gelatin capsule twice daily, at 9 A.M. and

TABLE 1

Grouping of cows and rations fed during the digestion trial

Ration	Cow No.	Av. daily intake			Daily dry matter intake
		Grain[a]	Hay[b]	Silage[c]	
		(lb.)			
I	1427	12.0	.....	72.0	33.2
	1471	8.0	.....	55.0	24.4
II	1435	14.0	.....	58.0	30.3
	1446	12.0	.....	68.0	31.4
III	1478	16.0	13.7	34.0	37.8
	1249	18.0	13.0	26.0	36.4
IV	1392	14.0	14.2	30.0	35.1
	1469	14.0	13.0	22.0	31.4

[a] The grain for Ration I was ground shelled corn—for Ration II, ground shelled corn + 3.5% urea—for Ration III, ground shelled corn, and for Ration IV, regular herd mix (15% crude protein).

[b] Good alfalfa hay.

[c] Corn silage.

9 P.M. These hours were selected only for the sake of convenience. During the ten-day collection period, the cows remained in their respective stanchions and total fecal collections were made manually. The fecal grab samples were taken at 6 A.M. and 4 P.M. and composited over the ten-day period. Dry matter content of the daily fecal output was determined, for use in making the final composite. Portions of the daily fecal sample, for the first six days of the ten-day trial, were composited on a dry matter basis and analyzed separately, to determine the digestibility of the rations as compared to the ten-day total.

II. Hourly variations in the excretion of chromic oxide within a 24-hr. period. Three days following the digestion trial, and while the cows were still being given 5 g. of chromic oxide twice daily, fecal grab samples were taken at 8 A.M. and at 2-hr. intervals thereafter for a 24-hr. period. These samples were dried in a forced-air oven, and analyzed for dry matter and chromic oxide.

Since the excretion pattern of chromic oxide by dairy cows *(10)* and steers *(6)* has been shown to follow the same trend if similar rations are fed, another group of eight cows was selected for the study of the effects of administering 10 g. of chromic oxide once daily (9 P.M.) upon the hourly variations in excretion of this inert substance and the relationship of the excretion of crude fiber and chromic oxide. The procedure for this trial was similar to that previously given for the first trial. During the course of the study one cow, receiving Ration I, was removed because of a digestive disturbance which resulted in reduced feed consumption.

The chromic oxide content of the feces was determined by the method of Gehrke and Baker *(4)*. This method proved to be very rapid and to yield excellent recoveries. Other analyses were made according to accepted A.O.A.C. methods *(1)*.

RESULTS AND DISCUSSION

I. Digestion studies—Chromic oxide versus total collection for determining digestibility. The data for the digestibility of dry matter as determined by the two methods are shown (Table 2), along with the data obtained from the six-day total collection. Two comparisons can be made from these data: *(a)* the accuracy of the chromic oxide method in determining the digestibility of dry matter, as compared to the conventional method; and *(b)* the effect of the length of the sampling period, six days versus ten days, upon the digestion coefficient of dry matter.

TABLE 2

Coefficients of apparent digestibility of dry matter

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	65.4	63.6	63.7
	1471	71.1	73.6	72.5
II	1435	64.3	64.7	62.3
	1446	67.3	67.4	67.7
III	1478	70.9	67.0	72.4
	1249	67.4	69.3	70.0
IV	1392	69.2	68.4	67.2
	1469	69.8	65.6	73.3
	Mean	68.2	67.5	68.6

[a] Grab samples were taken for ten consecutive days.

An analysis of variance of the data, as outlined by Snedecor *(14)*, showed no significant difference between digestion coefficients obtained using the chromic oxide method and those for the ten-day total collection.

No significant differences were found between data of the six-day and the ten-day total collection periods. This is in agreement with other findings at this station *(2)*. However, one must keep in mind that the length of the preliminary period would be a major factor in determining the length of time to collect feces, in order to eliminate errors in sampling. The cows used in this study had been consuming their respective rations for several weeks and were maintained on a constant feed intake during the entire trial.

The coefficients of digestibility for the crude protein and crude fiber are presented (Tables 3 and 4, respectively). In determining the digestibility of protein and crude fiber, more variation existed between methods than in the case of dry matter digestibility. These differences, however, were not statistically significant. Except for ash, the methods studied gave coefficients of digestibility for nitrogen-free extract and ether extract with about the same degree of accuracy as was found for crude protein and crude fiber. Ash was somewhat more variable, as is normally the case.

From these results, the chromic oxide method was found to give coefficients of digestibility comparable to those obtained by the conventional method.

TABLE 3
Coefficients of apparent digestibility of crude protein

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	60.5	58.1	59.9
	1471	59.6	60.7	59.6
II	1435	65.7	64.5	64.1
	1446	67.6	65.9	68.1
III	1478	66.7	64.3	67.5
	1249	74.2	72.4	73.9
IV	1392	64.7	64.8	66.5
	1469	76.4	70.4	77.5
	Mean	66.9	65.1	67.1

[a] Grab samples were taken for ten consecutive days.

II. Hourly variation in the excretion of chromic oxide. Chromic oxide administered once daily. Smith *et al. (13)* found that the fecal output of grazing cows could be estimated with about the same degree of accuracy, whether the chromic oxide was administered once daily, or twice. Lassiter *et al. (12)*, working with steers in a standard digestion trial, found less hourly variations in the excretion of chromic oxide when chromic oxide was administered twice, as compared to once, daily. If some of the hourly variation in the excretion of this indicator could be eliminated by the number of times of administration, the error of sampling could likewise be reduced.

A graphic illustration of the variation in the excretion of chromic oxide by seven cows for a period of 24 hr. is contained in Figure 1. The data are presented as per cent of the chromic oxide in the grab sample, compared to the mean chromic oxide content of all the grab samples taken for each cow during the 24-hr. period. The grab samples were removed from the rectum of the cow at the time specified. Six of the seven cows showed the highest level of fecal chromic oxide between the hours of 12 NOON and 6 P.M., and the lowest content

TABLE 4
Coefficients of apparent digestibility of crude fiber

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	62.3	60.2	62.3
	1471	66.5	68.3	68.9
II	1435	53.6	61.6	60.3
	1446	57.7	60.0	64.3
III	1478	54.8	56.3	59.6
	1249	51.4	49.9	53.8
IV	1392	45.2	51.8	54.4
	1469	48.2	45.5	57.6
	Mean	55.0	56.7	60.1

[a] Grab samples were taken for ten consecutive days.

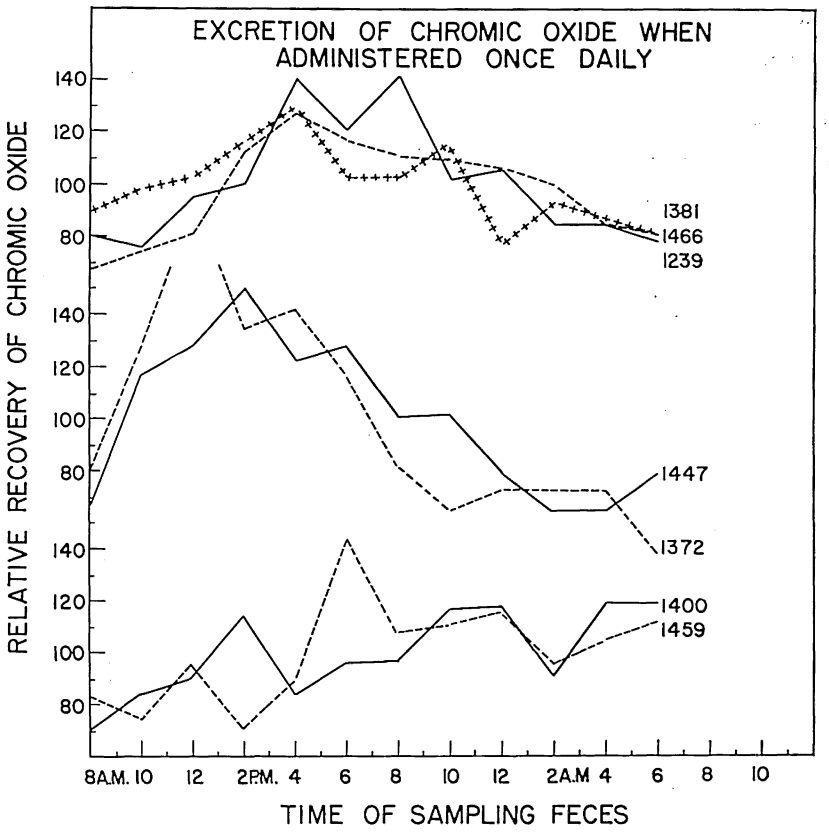


FIG. 1.

between the hours of 2 A.M. and 8 A.M. Five of the seven cows showed similar trends in the excretion of chromic oxide. The two remaining cows (No. 1400 and No. 1459) exhibited excretion curves different from the other cows, but similar to each other, in that the concentration of chromic oxide in the feces increased, instead of decreasing, after 4 P.M. Attempts to correlate the excretion curves of chromic oxide with dry matter intake and rations fed, failed to explain the cause of these variations. The average range in the rate of recovery of the chromic oxide from the feces was from 76 to 119%. Cow No. 1372 showed extreme variation (from 45 to 188%), but the excretion curve for this cow followed the same general trend as was found for cows showing less variation. The recommended time to grab sample *(6)*, to obtain samples which represented more nearly the mean chromic oxide content for a 24-hr. period, was found to be near 4 P.M. and 6 A.M. Using these two sampling periods, the mean per cent recovery of the chromic oxide for the seven cows was 102%, with a standard deviation of $\pm 4.3\%$.

Based on the findings of Crampton *et al. (3)*, in which poor recoveries of chromic oxide from the feces were obtained from feeding sheep an all-roughage

ration, an attempt was made to correlate hourly crude fiber excretions with those of chromic oxide. Crude fiber was determined on the grab samples taken for chromic oxide determination. The crude fiber content of the feces displayed a different excretion pattern and was less variable than that found for chromic oxide. Two of the seven cows (No. 1447 and No. 1239) excreted crude fiber at a uniform rate over the 24-hr. period. The mean crude fiber contents of the fecal grab samples were 28.0 and 30.9%, with a standard deviation of ±1.6 and ±1.4%, respectively. In general, the crude fiber excretion pattern agreed with the excretion pattern of lignin by cows, reported by Kane *et al. (10)*. This would be expected, because a large portion of the crude fiber fraction is composed of lignin. These results demonstrate that crude fiber and chromic oxide are excreted independently of each other, and that the diurnal variations in the excretion of chromic oxide are not associated with the excretion of crude fiber.

Administration of chromic oxide twice daily. The excretion of chromic oxide over a 24-hr. period when the chromic oxide was administered twice daily

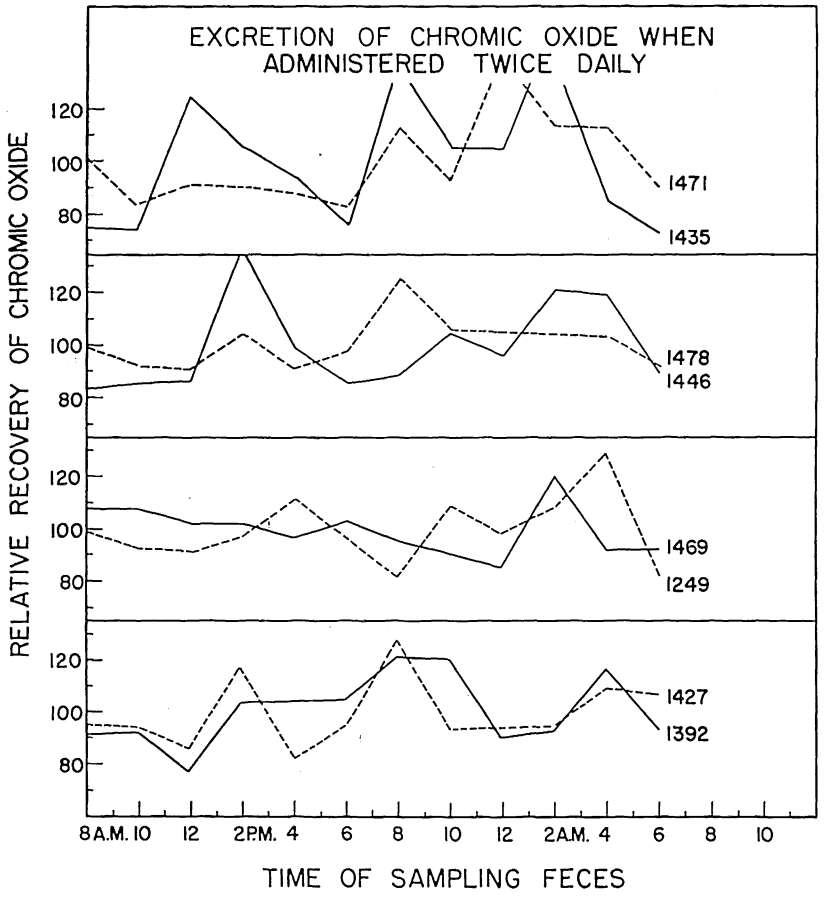


FIG. 2.

is shown (Figure 2). Data are presented as the per cent of the mean chromic oxide content of all fecal grab samples for each cow taken for the 24-hr. period. This enables one to make direct comparison of the excretion of chromic oxide in the feces of cows receiving different quantities of feed. Twice-per-day administration of chromic oxide appeared to lessen the variation in its excretion; however, considerable fluctuation still occurred. Even though less variation was noticed in hourly excretion rates of chromic oxide, the general excretion trend was erratic and would make it very difficult to devise a sampling plan in which the grab samples would give chromic oxide values near the daily mean value for all eight cows. Selection of the hours, 6 A.M. and 4 P.M., as previously used, results in an over-all mean recovery of 93% with a standard deviation of $\pm 4.1\%$. The range in per cent recovery for the eight cows, using these hours, was from 83 to 97%. Since these cows were used in the digestion studies in which the chromic oxide method gave satisfactory results, the data are indicative that there are day-to-day fluctuations in the excretion pattern of this indicator, which are overcome by sampling ten days.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Rockefeller Foundation.

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USE OF SODIUM PROPIONATE IN THE PREVENTION OF KETOSIS IN DAIRY CATTLE[1]

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SUMMARY

In a study involving 100 high-producing cows, one-half served as controls and one-half was fed 0.25 lb. of sodium propionate daily for a period of 6 wk., starting at calving. Blood analyses and milk production showed an advantage for the propionate-fed cows in terms of higher blood sugar levels, lower blood ketones, and higher milk production. Greatest differences were observed at 3 wk. after calving, when they were highly significant in favor of the propionate-fed cows in all three measurements. At 7 wk. after calving, after the propionate had been discontinued, there were no significant differences between the two groups. During the 6-wk. feeding period, nine cows in the control group showed sufficient symptoms of ketosis to require treatment; whereas, the level of propionate was increased on two cows in the propionate-fed group because of an abnormal blood picture.

The economic losses to the dairyman resulting from ketosis are well known. A recent review by Shaw *(13)* gives a comprehensive report of the history and present status of research on the problem.

Several fairly satisfactory treatments for ketosis have been developed. The ones most commonly being used in this country include glucose injection *(8)*, injection of glucocorticoids *(14)*, or the oral administration of sodium propionate *(9)*, propylene glycol *(6)*, or lactates *(13)*. Although these treatments, either alone or in combination, are reasonably satisfactory, prevention of the condition would be more satisfactory if it could be accomplished in a practical and economical manner.

Many suggestions have been made for the prevention of ketosis, but lack of controlled experiments makes them difficult to evaluate. The most common feeding practices recommended for this purpose include the feeding of molasses *(8)*, cobalt *(17)*, or high-protein rations *(15)*. The experimental evidence available *(13)* casts doubt on the value of molasses as an aid in ketosis prevention. No convincing experimental evidence has been presented to show that extra cobalt or protein above the amounts called for in our feeding standards is beneficial from the standpoint of ketosis. There seems to be fair agreement that such things as not having cows excessively fat at calving time, bringing them up to full feed rapidly after calving, and feeding high-quality roughage, are helpful in preventing ketosis, but they are based primarily on field observations which are difficult to evaluate.

Of the treatments, only those given orally seem to be very well adapted to use for prevention. After the initial report *(9)* on the use orally of sodium

Received for publication July 12, 1957.

[1] Supported in part by E. I. duPont de Nemours and Co., Inc., Wilmington, Delaware.

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propionate for the treatment of ketosis, it became obvious that this material might have some value in prevention. Other indications that it might be useful for this purpose came from observations that the introduction of propionic acid into the rumen of normal animals caused marked increases in blood sugar *(12)*, and that the level of propionic acid in the rumen was low at the time of ketosis *(2, 11)*.

The purpose of this study was to determine whether the feeding of sodium propionate for a period of 6 wk. after calving would prevent ketosis. The results of this study were reported upon earlier, in abbreviated form *(10)*.

EXPERIMENTAL PROCEDURE

The study covered a 3-yr. period and involved 100 cows (50 pairs) in three herds. Forty-four pairs of Holsteins, three pairs of Jerseys, and three pairs of Brown Swiss were used.

The cows within each herd were paired as equally as possible on the basis of previous production, history of ketosis, age, and time of calving. One member of the pair served as a control and the other, selected at random, was fed sodium propionate in the grain at a level of 0.25 lb. daily for a period of 6 wk., starting at calving.

In the case of 12 pairs, the propionate was measured out in a plastic cup for each cow at each feeding and mixed into the grain by hand. For the remaining pairs, the propionate was pelleted into part of the grain mix so that the final level was 2.5% of the special feed. By feeding 5 lb. of this special mix night and morning (10 lb. per day), an intake of 0.25 lb. of sodium propionate per cow per day was obtained. Any grain needed, in addition to the 10 lb., was made up of the regular grain mix without propionate. Within each herd all cows were fed the same roughage and grain mix and were handled normally. Grain was fed according to production.

Blood samples for sugar and ketone determinations were taken at 1, 3, 5, and 7 wk. after calving. The exact sampling date varied a maximum of two days from that scheduled, to permit twice-a-week sampling. Additional samples were taken if ketosis was suspected, and before and after treatment in the case of cows treated for ketosis. All blood samples were taken from the jugular vein. Methods for determining blood sugar and blood ketones were the same as those described in previous papers *(9, 12)*. Milk production was recorded on the day of sampling (in one herd, on the day before sampling). All cows were milked twice daily.

When complicating difficulties were encountered in one member of a pair, the pair was removed from the experiment. Metritis was the most common complication. Milk fever cases were not removed from the experiment, because they all recovered satisfactorily after treatment. Two of the experimental cows failed to eat the propionated feed well, so the pairs involved were removed from the experiment. Both of these cows had a history of being finicky eaters.

Ketosis was diagnosed only when all of the following symptoms were observed: *(1)* the cow was off feed, *(2)* production was down, *(3)* blood sugar was

low and blood ketones were high. Cows with ketosis were treated with sodium propionate. It was given by drench at a level of 0.50 lb. per day for as long as necessary, usually from five to ten days. All cows responded satisfactorily.

RESULTS

Table 1 gives a summary of the blood sugar, blood ketone, and milk production information on the 50 pairs of cows at the regular sampling intervals of 1, 3, 5, and 7 wk. after calving.

TABLE 1

Effect of feeding 0.25 lb. of sodium propionate daily for 6 wk. after calving, on blood sugar, blood ketones, and milk production (50 pairs of cows)

Time after calving	Control		Propionate-fed		Mean difference
	Mean	Range	Mean	Range	
1 Week					
Blood sugar *(mg.%)*	44.5	29.2-59.0	46.9	29.6-65.4	2.4
Blood ketones *(mg/%)*	11.0	1.0-35.0	8.9	2.6-19.9	2.1[a]
Milk production *(lb/day)*	51.2	28.1-70.2	54.4	34.6-73.0	3.2
3 Weeks					
Blood sugar *(mg%)*	42.8	25.0-63.6	48.0	24.6-59.2	5.2[b]
Blood ketones *(mg. %)*	15.7	3.5-46.5	9.9	3.8-23.9	5.8[b]
Milk-production *(lb/day)*	55.7	34.2-72.8	62.0	35.8-80.7	6.3[b]
5 Weeks					
Blood sugar *(mg. %)*	46.4	24.3-66.3	50.0	37.5-63.2	3.6[b]
Blood ketones *(mg. %)*	13.8	3.2-49.4	10.4	3.2-25.2	3.4[a]
Milk production *(lb/day)*	58.3	29.1-80.6	62.9	28.6-91.5	4.6[b]
6 Weeks — Propionate feeding discontinued					
7 Weeks					
Blood sugar *(mg. %)*	50.2	37.9-66.7	50.2	37.1-59.5	0
Blood ketones *(mg. %)*	10.5	4.2-32.4	8.5	3.5-26.7	2.0[a]
Milk production *(lb/day)*	59.1	33.6-83.5	60.0	18.1-93.0	0.9

[a] Differences significant ($P < 0.05$).

[b] Differences highly significant ($P < 0.01$).

It can be seen that the mean blood sugar level in the case of the control cows declined to a low point 3 wk. after calving and did not reach what are considered normal levels until 7 wk. after calving. Blood ketones showed the typical reciprocal relationship. In the case of the propionate-fed cows, the mean blood sugar levels were consistently higher and increased gradually, reaching normal levels at 5, rather than 7, wk. after calving. The mean blood ketone levels of the propionate-fed cows were consistently lower and reached a peak at 5, rather than 3, wk. after calving.

In the case of average milk production, the control cows showed a gradual increase in production, with maximum average production not being reached until 7 wk. after calving, when the blood sugar was normal and the blood ketones approached normal levels. The lower-producing cows appeared to reach their peak production earlier than the higher producers, which were more susceptible to ketosis. Average daily production of the propionate-fed cows was consistently higher, reaching a peak at 5 wk. and declining slightly at 7 wk. after the propionate was removed.

The mean differences between the control and propionate-fed cows in all three measurements were small at first, reached a maximum 3 wk. after calving, and essentially disappeared after the propionate was discontinued. In the case of blood sugar, the small difference in favor of the propionate-fed group 1 wk. after calving was not statistically significant. The differences increased, to become highly significant at 3 and 5 wk. after calving, then declined to zero at 7 wk. Blood ketones were significantly lower for the propionate-fed cows at 1, 5, and 7 wk. after calving, whereas at 3 wk. after calving the difference was highly significant. Differences in milk production were small and non-significant at 1 wk., became highly significant at 3 and 5 wk. after calving, and essentially disappeared after the propionate was discontinued.

The ranges in values (Table 1) show that the extremes were not too different in the two groups, except that blood ketones in the propionate-fed cows did not go to the extremely high levels observed in some of the control cows. Although the average blood sugar level of the propionate-fed cows was higher, the propionate feeding did not prevent a blood sugar drop in some of the cows.

Effect of the propionate feeding on the incidence of ketosis and the extent of the changes in blood sugar and blood ketones are shown (Table 2). There were nine cases of ketosis treated in the control group, whereas the level of propionate was increased to ⅜-lb. daily on two cows in the propionate-fed group. This was done because the blood picture was abnormal and milk production was down, although the cows were not off feed.

TABLE 2

Effect of feeding 0.25 lb. sodium propionate daily for 6 wk. after calving on the incidence of ketosis and the extent of changes in blood sugar and ketones (50 pairs of cows)

	Control	Propionate-Fed
Number of cases of ketosis treated	9	Level increased for 2 cows
Number of cows dropping below following levels blood sugar:		
40 mg/100 ml.	23	7
35 mg/100 ml.	14	2
30 mg/100 ml.	7	1
Number of cows increasing above following levels of blood ketones:		
20 mg/100 ml.	18	8
25 mg/100 ml.	12	1
30 mg/100 ml.	7	0
Mean minimum blood sugar *(mg/100 ml.)*[a]	39.1	43.8
Time of minimum sugar in days after calving	16.4	18.1
Mean maximum blood ketones *(mg/100 ml)*[a]	19.1	12.8
Time of maximum ketones in days after calving	22.4	21.6

[a] Differences highly significant ($P < 0.01$).

Table 2 also shows that the feeding of propionate reduced the number of cows showing an abnormal blood picture. Roughly 40% of the control cows showed at least a somewhat abnormal blood picture (below 40 mg. % sugar, above 20 mg. % ketones) some time during the first 6 wk. after calving. About half of these were not treated, because other symptoms were not serious enough.

The differences between the control and propionate-fed cows in minimum blood sugar and maximum blood ketone levels were both highly significant statistically in favor of the propionate-fed cows. The average times at which minimum sugar and maximum ketones were reached apparently were not influenced by the treatment.

Table 3 gives the blood sugar, blood ketones, and milk production of the cows developing ketosis and the borderline cases, at the various sampling intervals. All cows with blood sugar levels below 35 mg. % and blood ketones above 20 mg. %, at some time during the first 6 wk. after calving, were considered borderline. The reason they were not treated was that they were not reported to be off feed by the respective herdsmen.

It can be observed (Table 3) that all of the ketosis cases occurred between 1 and 5 wk. after calving. Most of these cases were slow in developing and could be predicted from the blood samples taken 2 wk. previously. The data on the two cows on which the propionate level was increased suggest that a level higher than ¼-lb. daily would be beneficial for certain cows.

The information on the borderline ketosis cases (Table 3) shows that cows can have a blood picture definitely indicating ketosis and still be eating and milking fairly well. Some of the borderline cows had a more abnormal blood picture than some of the treated cows. It is certainly likely, however, that these borderline cows would have responded to treatment with substantial increases in production. The highest producing cow in the whole study was the propionate-fed Cow 16H, with borderline ketosis, as shown in Table 3. She was milking over 80 lb. daily with a blood sugar level of less than 25 mg. % and was eating very well. She did, however, go over 90 lb. when the blood sugar returned to more normal levels.

Several of the propionate-fed cows that were apparently susceptible to ketosis dropped noticeably in production when the propionate was removed, but none developed ketosis.

No breakdown of the data by herd or by year is included. A statistical analysis of the blood sugar values showed that there were no significant differences due to herd or year. Services per conception were essentially the same for both treatments.

DISCUSSION

Results of this experiment appear to have both practical and theoretical significance. From a practical standpoint, it was shown that under the conditions of this experiment, it was possible to maintain higher blood sugar levels, lower blood ketones, and higher milk production, as well as to reduce the incidence of ketosis, by feeding sodium propionate. However, the fact that some of the propionate-fed cows showed a somewhat abnormal blood picture indicates that 0.25 lb. daily will not maintain a normal blood picture on all cows. Even at increased levels it is doubtful that it will prevent ketosis in all cows, because of the complicated nature of the condition. Certainly, its limitations under field conditions, where numerous complications are involved, should be recognized.

TABLE 3

Blood sugar, blood ketones, and milk production of cows developing ketosis and of borderline cases

Pair[a]	Weeks after calving: 1			3			5			7		
	Sugar	Ketones	Milk	Sugar	Ketones	Milk	Sugar	Ketones	Milk	Sugar	Ketones	Milk
	(mg. %)	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*
Control cows developing ketosis												
1H	45.9	10.1	45.0	38.3	20.2	47.9	30.2	49.4	29.1[b]	49.0	17.4	52.4
9H	46.5	8.6	63.0	49.6	6.2	72.8*	50.7	5.5	80.6	38.3	16.0	63.7[c]
10H	50.0	4.9	50.4*	55.2	4.4	60.5	52.8	5.3	59.9	56.2	6.5	63.4[c]
27H	47.9	12.2	68.4	29.6	28.1	68.6[b]	37.5	32.9	72.7	43.4	18.0	83.5
28H	39.9	15.2	60.8	35.5	26.0	60.0*	46.5	14.4	68.2	52.8	6.9	65.5[c]
30H	36.9	17.5	31.4	25.7	29.3	40.2[b]	44.4	12.7	58.4	44.3	11.9	58.8
34B	40.3	22.5	42.7	28.1	46.5	37.7[b]	40.2	42.7	45.0	46.5	12.1	48.5
40H	31.6	35.0	59.7[b]	32.1	25.1	61.6	46.5	21.3	58.6	43.9	16.2	63.8
50H	37.7	25.8	54.8	26.9	42.7	46.5[b]	51.4	6.2	77.0	56.6	4.2	80.5
Propionate-fed cows for which level of propionate was increased												
30H	40.1	16.8	54.0	38.9	22.8	56.1	37.5	25.2	53.4[b]	50.4	5.7	64.7
50H	29.6	12.2	65.1[b]	45.0	5.9	68.2	56.2	3.4	82.5	56.4	4.2	79.2
Borderline[d] control cows not treated because not off feed												
4H	40.9	21.8	55.3	34.3	23.0	56.5	46.0	14.5	62.8	46.0	8.7	62.1
5H	33.5	13.9	60.1	31.1	19.9	64.3	37.1	30.8	60.9	37.9	19.1	70.1
14H	29.2	18.5	51.4	27.1	36.7	57.0	24.3	32.4	57.4	48.9	32.4	69.4
25H	36.9	8.9	63.0	25.0	37.0	59.0	34.5	30.1	74.0	42.8	22.6	77.2
37H	40.3	8.3	61.3	33.2	26.4	60.0	46.0	16.4	72.1	42.4	14.5	73.3
45H	47.9	6.3	57.9	33.1	16.4	67.5	28.8	20.1	54.2	55.7	8.3	62.0
Borderline[d] propionate-fed cow not treated because not off-feed												
16H	37.1	11.0	73.0	24.6	20.7	80.7	43.4	19.4	91.5	48.9	12.4	93.0

[a] H = Holstein, B = Brown Swiss.

[b] Treated for ketosis at time indicated.

[c] Treated for ketosis between sampling intervals at time indicated by *.

[d] All cows with blood sugar levels below 35 mg. per 100 ml., and blood ketones above 20 mg. per 100 ml., considered borderline.

In this experiment, ketosis was controlled in both groups of cows. In one group it was by a prevention schedule, in the other by treatment of the cases as they developed. Less propionate was used for the treatment schedule, because only 18% of the cows were treated and the amount used per cow was somewhat less. However, the propionate-fed cows did produce approximately 200 lb. of milk per cow more during the 6-wk. feeding period. This increased production appeared to be owing to the fact that they were able to maintain production closer to normal rather than any stimulation above normal.

Under field conditions, whether or not a preventive schedule of this type would be practical would appear to depend upon several factors, such as the incidence of ketosis, the level of production, the price of milk, and the cost of propionate. It would appear to have its greatest value when used on selected cows, in high-producing herds with a high incidence of ketosis. In addition to their use for strictly preventive purposes, other possible uses of feeds containing propionate are as a treatment for mild cases of ketosis while the cow is still eating, or as a follow-up treatment, to prevent relapses.

Other observations of practical importance, aside from the specific objective of the experiment, included the following: *(1)* Most of the high-producing cows passed through a borderline ketosis period during the first 6 wk. after calving. Feeding and management practices during this period are critical. Oral ketosis-control measures offer a convenient way of handling these borderline cases that are still eating fairly well. The high average values for blood ketones suggest that care should be exercised in the interpretation of the qualitative urine test for ketosis during this period. Not all cows with a positive urine test require treatment. *(2)* The time of occurrence of the ketosis cases and the general trend of blood sugar and blood ketone levels showed that the problem period was limited to approximately the first 2 mo. after calving. This suggests that difficulties outside this period would not likely be ketosis. *(3)* Ketosis cases which occurred were slow in developing. In most cases, there was a gradual increase in blood ketones and a gradual decline in blood sugar, milk production, and appetite. Usually, the blood picture showed abnormalities 2 wk. before the cows were actually treated. This indicates that when a uniform system of feeding and management is followed during this period, and when the cows are observed carefully, cows going off in feed and milk abruptly are apt to have something other than ketosis as the primary cause of their difficulty. Abrupt changes in feeding or management, on the other hand, could rapidly throw the borderline cases into ketosis.

This work adds additional support to the idea suggested by the use of sodium propionate for treatment: that it might be possible to control ketosis by nutritional means. The theoretical reasoning behind it involves the concept that propionate serves as a glucose precursor, which is more effective orally than glucose itself, because it escapes bacterial action in the rumen and is absorbed directly. The manner in which propionate may fit into the scheme of intermediary carbohydrate metabolism after absorption has been reviewed by several workers *(1, 5, 7)*. The other three carbon compounds effective orally apparently follow

either the same ultimate pathway and serve as glucose precursors or, as is the case with lactic acid under most normal circumstances, are converted in large part to propionic acid before absorption *(4, 16)*. The fact that such a small amount of propionate gave as much response as was observed is somewhat surprising. The increased milk production cannot be completely accounted for by the propionate consumed. Apparently, it was not only a matter of conversion of propionate to glucose to lactose, but an effect on the general well-being of the cow in terms of increased efficiency of intermediary metabolism and/or increased feed intake.

One interesting possibility suggested by this work is that by feeding certain feeds or combinations of feeds, or by the use of special inoculum, one might cause increased production of propionic acid in the rumen during this ketosis-susceptible period, thus accomplishing the same results without adding propionate to the feed. It has been demonstrated by several workers that the proportions of volatile fatty acids in the rumen may be changed by alterations in the diet *(3, 13)*. Work on this aspect of nutritional control of ketosis is under way.

ACKNOWLEDGMENT

The author wishes to acknowledge the cooperation of Dr. L. S. Mix of the Beacon Milling Company, Cayuga, New York, in giving suggestions, preparing special feeds, and in making the Beacon dairy research herd available for use on this study. The cooperation of Dr. J. K. Loosli in making animals available and in giving suggestions is also acknowledged.

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EFFECT OF FEEDING SODIUM PROPIONATE ON MILK AND FAT PRODUCTION, ROUGHAGE CONSUMPTION, BLOOD SUGAR, AND BLOOD KETONES OF DAIRY COWS [1]

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SUMMARY

A double-reversal experiment involving ten cows from the Cornell University Dairy Herd was used to detect the effect of the daily feeding of ½ lb. of sodium propionate on milk production, butterfat test, hay consumption, blood sugar and blood ketone levels. There were no significant differences ($P < 0.05$) in the milk production, hay consumption, blood sugar levels, blood ketone levels, or weight gains or losses attributed to the feeding of sodium propionate. There was a slight but nonsignificant fall in butterfat test.

In a supplemental experiment, two fistulated steers were fed propionate and control feed, and the fatty acid content of the rumen was determined at regular intervals after feeding. The feeding of the propionate feed resulted in higher levels of all the volatile fatty acids in the rumen for the first 3 hr. after feeding, particularly the propionic. From 3 to 9 hr. after feeding, the concentrations of rumen acids from the two feeds were quite similar. It was calculated that all of the administered propionate had disappeared in 7 hr., and most of it in 3 hr.

Sodium propionate has been shown to be effective in the treatment and prevention of ketosis. Schultz *(9)* found that the oral administration of ½ lb. of sodium propionate daily for from three to ten days to cows with uncomplicated ketosis was effective. Schultz *(10)* also found that the daily feeding of ¼ lb. of sodium propionate to cows for 6 wk. after calving reduced the incidence of ketosis. The propionate-fed cows showed higher blood sugar levels, lower blood ketone levels, and produced more milk for the first 6 wk. after calving than did the control cows.

Because of the stimulation in production observed when sodium propionate was fed or administered to cows with mild to severe ketosis, it was felt desirable to determine the effect of feeding it to normal cows. Information on this point was requested by those associated with official testing programs of the breed associations.

Tyznik and Allen *(11)* found that cows having a low butterfat test due to a high concentrate–low roughage diet showed a decreased percentage of acetic acid in the rumen, an increased percentage of propionic acid, and a normal percentage of butyric acid. When acetate was fed to these cows, the butterfat tests returned to normal. Miller and Allen *(7)* reported that the daily feeding of 1 lb. of sodium acetate to normal cows did not affect significantly the milk production, fat test, blood sugar or ketone values.

Received for publication July 17, 1957.

[1] The data published in this paper are from a thesis presented by the senior author to the Graduate School, Cornell University, in partial fulfillment of the requirements for the degree of Master of Science, February, 1956.

This experiment was designed to determine whether the feeding of sodium propionate would affect the milk production, fat test, roughage consumption, blood sugar and ketone levels of normal cows.

EXPERIMENTAL PROCEDURE

Eight Holstein and two Brown Swiss cows from the Cornell University Dairy Herd were used in this study. These cows were paired according to breed, age, and previous production. The cows were placed on the experiment on the 2nd wk. after calving. The 1st wk. after calving served as a preliminary period.

A double-reversal experiment was used, consisting of six experimental periods. Each period consisted of two weeks, with no transitional period between the experimental periods. Two types of grain were fed during the experimental periods. The first type was an 18% protein pelleted grain mix containing 5% sodium propionate. Poundages of composition were as follows: linseed oil meal and soybean oil meal, 200 each; gluten feed, distillers grains, and sodium propionate, 100 each; wheat bran, 300; ground oats, 500; hominy, 450; salt, 20; calcite, 10; dicalcium phosphate, 15, and trace minerals, 5. The other type of grain was the same mixture, except that the propionate was replaced by hominy feed. This served as the control feed.

One member of each pair was started on the control feed and the other member on the propionate feed. At the end of 2 wk. the type of grain was changed, and this was repeated every 2 wk. until a cow had been on three experimental periods of propionate feed and three periods of control feed.

Each cow received 10 lb. of propionate or control feed daily. During the propionate periods each cow received ½ lb. of sodium propionate from these 10 lb. of grain. The amount of grain required was determined from Appendix Table VIIIa in Morrison *(8)*. The difference between the 10 lb. of the basal grain mix fed and the total amount of grain required daily was made up of the regular herd mix containing 16% protein. The composition of this in pounds was as follows: ground oats, 460; ground corn or hominy, 400; wheat bran, 300; corn gluten feed, 500; soybean oil meal, 200; cane molasses, 100; iodized salt and steamed bone meal, 20 each; also, there was 2 g. of cobalt salt.

The amount of grain fed was changed at 4-wk. intervals, to insure that the same amount of grain was fed during one period of propionate feeding and one control period. An upper limit of 20 lb. of grain daily was set for all cows, to insure complete grain consumption. Each cow received 36 lb. of corn silage daily, and a mixed hay containing approximately 30% legume was fed ad libitum. Daily weigh-backs were taken on all feed, to insure accurate records of feed consumption.

Daily milk weights were taken and weekly composite milk samples were analyzed for butterfat content by the Babcock method. Blood samples were taken from the jugular vein at weekly intervals.

Potassium oxalate was used to prevent coagulation of the blood. The proteins were precipitated by the method described by Folin and Wu *(5)* and the blood sugar was determined colorimetrically by the method of Benedict

(2). Blood ketones were distilled from the filtrate by the method of Behre and Benedict *(1)* and the ketone bodies were determined as acetone by the method of Block and Bolling *(3)*.

Three-day weights were taken on all cows before they started on the experiment and before they were switched from one feed to another.

In a supplemental experiment, two dairy steers with rumen fistulas were used to determine the rate of disappearance of the propionic acid from the rumen. The steers were fed 5 lb. of control feed and 5 lb. of hay twice daily for 1 wk. At the end of a week, the fatty acids in the rumen were determined. A rumen sample was taken in the morning before the animal was fed and at hourly intervals after feeding for 10 hr. The 2nd wk., the steers received the same amount of propionate feed and hay, and at the end of the 2nd wk. the fatty acids in the rumen were determined by the same method.

The rumen samples were processed, distilled, and evaporated by the method described by Card *(4)*. The volatile fatty acids were separated according to the procedure of Marvel and Rands *(6)*, as modified by Card *(4)*.

FIG. 1. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on milk production and butterfat test.

RESULTS AND DISCUSSION

The average daily milk production by weeks is given (Figure 1). The daily feeding of ½ lb. of sodium propionate did not affect the milk production. The cows produced an average of 53.6 lb. of milk daily on the control feed and 53.5 lb. on the propionate feed. These results indicate that the stimulation in milk production observed following the feeding of propionate to ketosis cows was a result of a return to normal rather than a stimulation above normal.

A slight reduction in the butterfat tests occurred on the propionate feed. The weekly butterfat tests are also given (Figure 1). The feeding of propionate caused an average reduction of 0.11% in the butterfat tests during the entire experiment (Table 1). Analysis by the "t" test showed that this was not statistically significant. The high peak in the butterfat test during the 2nd wk. of control feeding was the result of two cows having exceptionally high butterfat tests. The butterfat tests for the first 6 wk. were more variable than for the last 6 wk.

TABLE 1

Summary of the effects of feeding one-half lb. of sodium propionate daily to dairy cows during alternate 2-wk. periods after calving

Measurement	Average values[a] for three 2-week periods on: Control feed	Propionate feed
Milk production *(lb/cow/day)*	53.6	53.5
Milk fat test *(%)*	3.98	3.87
Total fat *(lb/cow/period)*	30.1	29.1
Hay intake *(lb/cow/day)*	14.6	14.6
Silage intake *(lb/cow/day)*	35.0	35.3
Grain intake *(lb/cow/day)*	17.3	17.3
Blood glucose *(mg. %)*	50.0	50.7
Blood ketones *(mg. %)*	5.8	4.8
Body weight changes *(lb/cow/period)*	+0.1	−0.8

[a] Differences due to propionate were not statistically significant in the case of any of the measurements.

The effect of propionate feeding on blood sugar and blood ketone levels is shown (Figure 2). The general conclusion that can be drawn is that when the blood glucose and ketone values were normal, propionate feeding caused no appreciable change. However, when these values varied from the normal in the direction of ketosis, propionate feeding tended to cause them to return to normal. During the first 2-wk. period, the cows started on the control feed showed above-normal blood ketone levels and slightly low blood sugar values. The milk fat test was also unusually high (Figure 1). The feeding of propionate during the next 2-wk. period appeared to bring all of these measurements back toward normal. During the rest of the experiment, the blood sugar and ketone values were within the normal ranges and propionate feeding caused no appreciable changes.

The average values of the various measurements taken during the course of the experiment are given (Table 1). Milk production and feed consumption were practically identical on the control and propionate feeds. Milk fat percentage and total fat production were reduced by the propionate feeding, but the differences were not statistically significant. Average blood ketone values were slightly lower and there was a very slight weight loss while the cows were being fed the propionate. Again, the small differences were not statistically significant. Most of the cows lost weight during the first 6 wk. of the experiment and gained during the last 6 wk.

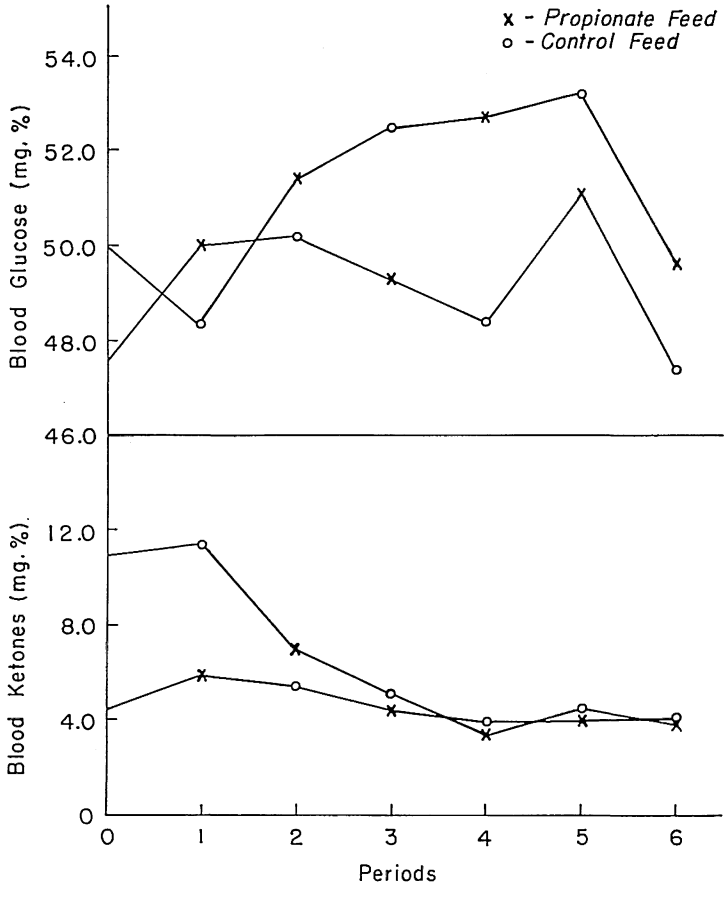


FIG. 2. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on blood sugar and blood ketones.

The results of the supplemental experiment, designed to determine what happened to the fed propionate and how it affected the normal rumen fermentation, appear in Figure 3. The control feed showed the normal curve of fatty acid content in the rumen following feeding, with peak concentration 3 hr. after feeding. When the propionate feed was fed, the level of propionic

acid logically was much higher when the first sample was taken 1 hr. after feeding. It is of interest to note that the levels of acetic and butyric acid were also higher. It is doubtful whether this could be interpreted to mean that the propionate stimulated the production of the other acids, but certainly

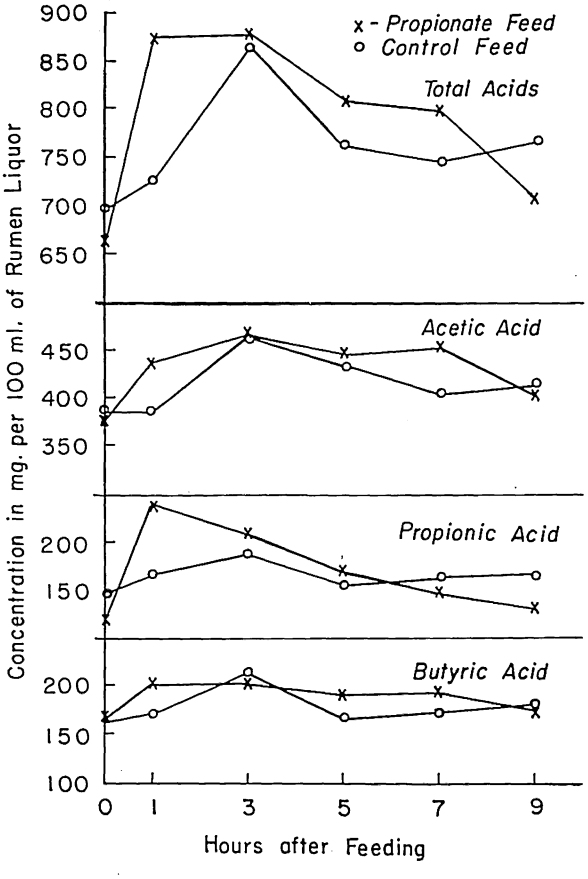


FIG. 3. Effect of feeding 5 lb. of grain containing 5% sodium propionate on volatile fatty acid concentration in the rumen.

it is clear that it did not depress their normal production. After the 3-hr. peak concentration was reached in each case, the curves for the control and propionate feeds followed each other closely. It was calculated that all the administered propionate had disappeared in 7 hr., with most of it disappearing within 3 hr.

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GROUND CORN COBS AS A SOURCE OF ROUGHAGE FOR LACTATING DAIRY COWS[1]

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SUMMARY

Two studies were conducted with milking cows to compare ground corn cobs with alfalfa hay for roughage, and a high protein (20.8 to 22.6%) grain mix was fed to supply Morrison's *(9)* minimum TDN standards, assuming that the corn cobs had the same TDN value as the alfalfa hay. In Trial I, corn cobs were substituted for hay at the rate of 8.0 and 16.0 lb. per day without affecting milk production, fat test, or body weight. An alfalfa-brome grass hay, which had a TDN value of 47.8%, was used for comparison.

In Trial II, corn cobs replaced alfalfa hay up to 60% of the total roughage intake without affecting milk production, and up to 80% of the total roughage without seriously affecting milk production. The TDN content of the alfalfa hay was 52.0 to 53.0%. When the cows were fed 0.4 lb. of hay and 2.1 lb. of corn cobs per 100 lb. of body weight, consumption of total roughage decreased 1.6 lb. per cow daily. Corn cobs appear to be equal to medium-quality alfalfa hay up to the limit of the cow's ability to consume them. Digestion trials were conducted in both studies, but no significant trends were noted.

The use of ground corn cobs as a roughage for beef cattle has been well established *(1, 2, 3)*. Fries *et al. (4)* of the Kentucky station and Harshbarger *(6)* of the Illinois station have reported data showing that corn cobs can be used as a satisfactory roughage for growing heifers. Hill *et al. (7)* of the Purdue station reported data which indicated that corn cobs were not a satisfactory source of roughage for milking cows. Cows fed corn cobs as the sole source of roughage declined 53.4% in milk production over a 12-wk. trial, as compared to a 41.5% decline for cows fed alfalfa hay and silage as the roughages. Both groups were fed "Purdue Supplement A" as the grain mixture. Cows fed corn cobs, however, gained 93.3 lb. in weight as compared to 16.0 lb. for the alfalfa hay and silage cows. The total dry matter consumption per 100 lb. of body weight was 2.36 lb. per day for the cows fed corn cobs, and 2.76 lb. for the alfalfa hay and silage fed cows. Graf and Engel *(5)* of the Virginia station reported the feeding of a limited amount of corn cobs, 5.5 lb. per day, in place of an equivalent amount of alfalfa-grass hay. The same amount of corn silage and grain was fed to each group. Cows fed no corn cobs produced 22.0 lb. of milk more per group than the cows receiving corn cobs, over a 30-day period. Cows fed corn cobs gained slightly more in weight than the cows fed hay. Digestion trial data favored slightly the cows fed corn cobs.

Received for publication September 3, 1957.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2126.

The purpose of this experiment was to further evaluate the use of ground corn cobs as a source of roughage for milking cows, and particularly to determine the extent to which corn cobs could be used as a substitute for hay.

EXPERIMENTAL PROCEDURE

Two studies have been made concerning the use of ground corn cobs as the source of roughage for milking cows.

Trial I. Fifteen lactating Holstein cows were used in five 3 × 3 Latin-square designs. Each period was 27 days in length. Group 1 received 25.0 lb. per day of a second-cutting alfalfa-brome grass hay; Group 2 received 17.0 lb. of hay and 8.0 lb. of corn cobs, and Group 3 received 9.0 lb. of hay and 16 lb. of corn cobs. The total digestible nutrient (TDN) content of the hay was 47.8%. All cows were fed a simple grain mixture composed of 1,260 lb. of yellow corn, 700 lb. of soybean oil meal, 20 lb. of trace salt containing idoine and cobalt, and 20 lb. of dicalcium phosphate. Grain was fed at a rate to supply Morrison's *(9)* minimum TDN standards. Adjustments in grain feeding were made at the end of each period based upon the average decline in milk production of all cows *(8)*. Milk weights were recorded daily and samples for butterfat percentage were taken from each milking and composited on a three-day basis. Digestion trials were conducted with seven cows receiving comparable amounts of feed during each of the three periods, using chromic oxide as the indicator. Grab samples of feces were collected twice daily, at 6 A.M. and 4 P.M. for seven days after the cows had been on the rations for a minimum of ten days. All cows were weighed every third day throughout the study.

Trial II. Sixteen lactating cows (12 Holsteins and four Jerseys) were divided into four comparable groups and used in 4 × 4 Latin-square designs. Each period was 28 days in length, to compare four levels of hay and corn cobs as roughages for milking cows. All cows were fed roughage at a rate of 2.5 lb. per 100 lb. of initial body weight. The rations were as follows: Group 1—2.5 lb. of hay per 100 lb. of body weight; Group 2—1.8 lb. of hay and 0.7 lb. of corn cobs per 100 lb. of body weight; Group 3—1.1 lb. of hay and 1.4 lb. of corn cobs per 100 lb. of body weight, and Group 4—0.4 lb. of hay and 2.1 lb. of corn cobs per 100 lb. of body weight. Two alfalfa hays were used in the study. During Periods 1 and 2, a prebloom alfalfa hay with TDN content of 53.0% was used, and during Periods 3 and 4, an excellent quality third-cutting alfalfa hay with a TDN content of 52.0%. A grain mixture composed of 1,210 lb. of yellow corn, 750 lb. of soybean oil meal, 20 lb. of a trace salt containing iodine and cobalt, and 20 lb. of dicalcium phosphate was fed as outlined in Trial I. Body weights were recorded for three consecutive days at the beginning and at the end of each period. Daily milk weights were recorded and samples for butterfat percentage were taken from four consecutive milkings each week. Digestion trials were conducted on all cows during the last seven days of each period, according to the procedure used in Trial I. The corn cobs in both studies were ground through either a 0.625 or a 0.5 screen.

RESULTS AND DISCUSSION

Composition of the experimental feeds for both Trials I and II is shown (Table 1). In Trial I, the hay was 0.5 bloom alfalfa-brome and was slightly rain-damaged, which accounts for its slightly low TDN value. The grain mixtures

TABLE 1
Composition of experimental feeds

Feed	Dry matter	Crude protein	Crude fiber	Ether extract	N-free extract	Ash	TDN[a]
				(%)			
				Trial I			
Alfalfa-brome hay	88.1	13.4	34.7	1.2	33.0	5.8	47.8
Corn cobs	81.4	3.3	25.6	0.8	50.0	1.2	.....
Grain mix	87.1	20.8	3.8	3.1	54.8	4.6	.....
				Trial II			
Alfalfa hay[b]	89.2	17.6	28.1	1.9	34.6	7.0	53.0
Alfalfa hay[c]	84.4	15.3	28.1	1.4	33.8	5.8	52.0
Corn cobs	84.1	2.0	30.3	0.2	50.2	1.4	.....
Grain mix	87.9	22.6	3.0	2.7	54.5	5.1	.....

[a] Actual TDN values.
[b] Used in first part of Trial II.
[c] Used in last part of Trial II.

in both Trials I and II were calculated to supply enough protein to meet the cows' requirements when receiving the least amount of hay and the largest amount of corn cobs; thus, when the cows were fed a high proportion of hay, an excessive amount of protein was fed.

The consumption of feed in both trials is presented (Table 2). None of the differences in roughage or grain consumption among groups was statistically

TABLE 2
Summary of feed consumption
(Av. intake/cow/day)

Ration	Hay[a]	Corn cobs[a]	Grain[a]
		Trial I	
A	24.9	.....	8.7
B	17.0	7.8	8.7
C	9.0	15 3	8.5
		Trial II	
1	27.8	.....	6.6
2	20.1	7.7	6.6
3	12.2	14.9	6.7
4	4.5	21.7	6.8

[a] No significant differences.

significant; however, as the level of corn-cob feeding increased, there was a tendency for the total consumption of roughage to decrease. Cows on Ration C (Trial I) consumed a total of 24.3 lb. of roughage per day, compared to 24.9 lb.

for Ration A and 24.8 lb. for Ration B. In Trial II, the cows on Ration 4 consumed only 26.2 lb. of total roughage per day, compared to 27.1 lb. for Ration 3, and 27.8 lb. for both Ration 1 and Ration 2. The decrease in corn-cob consumption was not the result of a few cows refusing to eat corn cobs but of a general lowering of corn-cob consumption by all cows. The grain mixtures used in these studies did not include molasses, which may have improved the consumption of corn cobs.

Effect of the various levels of corn-cob feeding on milk production and body weight changes is presented (Table 3). The feeding of corn cobs did not appear to have any appreciable effect on the average butterfat percentage in any trial.

TABLE 3

Effect of feeding corn cobs as a partial replacement of hay on the milk production and body weight changes of dairy cows

Ration	Av. test[a]	Av. 4% FCM[a]		Average body weight change[a]
		7 to 28 days	Last 7 days	
	(%)	*(lb/day)*		*(lb/cow)*
		Trial I		
A	3.5	33.1	32.6	—3.7
B	3.5	33.8	34.3	9.0
C	3.7	33.6	33.7	4.7
		Trial II		
1	4.0	29.2	28.4	9.9
2	3.9	28.3	27.8	3.7
3	4.0	28.6	27.7	22.2
4	4.1	27.2	26.2	16.4

[a] No significant differences.

This was true even with the cows fed Ration 4 (Trial II), when only 4.5 lb. of hay was consumed daily. In Trial I, the feeding of either 8 or 15 lb. of corn cobs per day did not significantly affect milk production. Similar observations were made in Trial II; however, the milk production of cows fed Ration 4 was lower than the other three groups, even though these differences were not statistically significant. The feeding of corn cobs did not significantly affect body weight changes, although the cows fed high amounts of corn cobs tended to make the greatest body weight gains. This observation is in agreement with both the Purdue *(7)* and Virginia *(5)* workers.

Data (Table 4) show the effect of the various corn-cob rations on the over-all digestibility of the rations. In Trial I, the digestibility of protein was significantly higher in Ration A than in either Ration B or Ration C. Possibly, this could be due to the higher level of protein intake in this ration. Crude fiber digestion was higher with Ration C than with either A or B, but these differences were not significant. Dry matter digestibility did not appear to be influenced significantly.

In Trial II, the coefficients of digestibility were similar to those obtained in Trial I. No significant differences existed in crude fiber digestibility among

rations, but the dry matter coefficients for Rations 1 and 2 were higher than for Rations 3 and 4. The coefficients obtained for Ration 3 were higher than those for Ration 4. Similar trends were noted for crude protein digestion which were probably due to a lower intake of crude protein in Rations 3 and 4. No significant difference existed among rations in respect to crude fiber digestibility coefficients.

TABLE 4

Effect of various levels of corn-cob feeding on the apparent digestibility of the over-all ration

Ration	Dry matter	Crude protein	Crude fiber	Ether extract	N-free extract
	(%)				
			Trial I		
A	56.6	65.0	45.4	35.1	65.0
B	52.2	59.9	44.6	34.8	58.4
C	56.2	58.7	51.8	48.2	59.7
L.S.D. —5%	NS	4.39	NS	.....	.....
			Trial II		
1	61.3	67.5	41.7	55.3	67.1
2	60.0	67.2	43.4	56.2	62.8
3	57.3	59.7	37.8	57.2	60.5
4	54.9	44.5	37.8	56.3	58.4
L.S.D. —1%	.....	3.70	NS	.....	.....
L.S.D. —5%	2.12	.....	NS	.....	.....

The results of these two studies concerning the nutritive value of ground corn cobs as a source of roughage for milking cows indicate that when corn cobs are properly supplemented, they contain more feeding value than is commonly recognized. Results of Trial I showed that corn cobs were equal to medium-quality hay when fed to the limit of substitution for hay used in this study. Trial II was conducted primarily to determine the extent to which corn cobs could substitute for hay as a source of roughage in the ration of milking cows, and also to compare corn cobs with a higher quality hay (TDN) than was used in Trial I.

Data obtained in Trial II show that corn cobs can be substituted up to about 60% of the total roughage intake without affecting milk production. It should be pointed out that a very high-quality hay (52.0-53.0% TDN) was used in Trial II and was substituted by corn cobs on an equal pound basis. This means that if a slightly poorer hay—which would probably be the quality of hay on most dairy farms—had been used as a comparison, corn cobs would have compared more favorably with hay. It appears that corn cobs are equal to average-quality hay on a per-pound basis up to the limit of the cows' ability to consume corn cobs. In Trial II, consumption of corn cobs was not equal to hay, when corn cobs composed 80% of the total roughage intake.

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GROWTH STUDIES WITH CALVES AND RABBITS FED TIMOTHY HAY GROWN ON HEAVILY FERTILIZED SOILS [1, 2]

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SUMMARY

This experiment was conducted to identify nutritional deficiencies in timothy hay grown on a heavily fertilized soil. When this hay was fed to calves with a concentrate mixture of degermed corn meal, nonfat dry milk solids or urea, vitamins A and D, cobalt, calcium, phosphorus, and common salt, the ration was deficient in iron, copper, and iodine, resulting in poor growth, poor appetite, anemia, and enlarged thyroids. Feeding iron and copper, either individually or in combination, did not correct these symptoms. Iodine supplementation corrected the thyroid condition but did not stimulate growth. When similar hay was fed to rabbits in a ration supplemented with wheat gluten, hydrogenated vegetable oil, dextrose, sodium chloride, and vitamins A and D, it also resulted in poor growth, anemia, and increased thyroid weight. Feeding of a ration containing 5% alfalfa prevented all these symptoms, but an equivalent amount of alfalfa ash or iodine prevented only the enlarged thyroid. The timothy hay apparently was deficient in iodine and some organic factor(s) which may be found in alfalfa hay.

An experiment which has been in progress at the New Hampshire Agricultural Experiment Station for a period of several years has shown that certain forage crops grown under conditions of heavy applications of NPK fertilizers to the soil may be low in iron, copper, cobalt, and magnesium. When such forages were fed to dairy calves, along with a low trace mineral concentrate mixture containing supplemental vitamins A and D, calcium, phosphorus, cobalt, and common salt, they resulted in poor growth, anemia, sagging of the spinal column behind the shoulders, and other symptoms. In mature animals, the feeding of this type of ration has resulted in rapid loss of flesh after calving, poor milk production and, in many instances, in the production of goitrous calves. Rabbits fed similar forages have shown poor growth, mild anemia, and hyperplastic thyroids.

Received for publication January 27, 1957.

[1] Published with the approval of the Director of the New Hampshire Agricultural Experiment Station, as Scientific Contribution No. 197.

[2] This study was supported in part by a research grant from Central Laboratories, General Foods Corporation, Hoboken, N. J. The vitamin A acetate was contributed by the Chas. Pfizer Co., Inc., Terre Haute, Ind., and the crystalline urea by E. I. duPont de Nemours and Co., Wilmington, Delaware. This study was carried out as a subsidiary phase of a project entitled "The Effect of Trace Mineral Deficiencies on the Reproductive Performance of Dairy Cattle." This main project was contributory to the regional research project, NE-1, entitled "Causes and Prevention of Reproductive Failure in Dairy Cattle."

While the need for supplemental cobalt under such conditions in New Hampshire has been well established *(1)*, a need for iron and copper has not. It was the purpose of the experiments reported here to determine whether iron and copper were lacking in the ration of calves, and to determine the responsiveness of rabbits fed these deficient rations. In supplementary studies iodine, zinc, and alfalfa were tested for growth-stimulating properties.

EXPERIMENTAL PROCEDURE

Calf studies. The forage studied in this experiment was first-cutting timothy hay, harvested in 1954 from a Paxton loam. It was seeded in 1950 on land which was limed before seeding and fertilized each year with a 7–7–7 mixture, applied at the rate of 600 lb. per acre in April and 400 lb. per acre after the first crop was removed. The hay was harvested in the early-bloom stage, cured and baled in the field.

The experimental animals used were 16 Guernsey heifer calves purchased from well-fed herds. The calves were kept in individual wooden pens and were bedded with shavings and watered from aluminum pails. In the pre-experimental or depletion period, the calves were weaned from whole milk or milk containing concentrates, to a hay and grain concentrate regimen, in the following manner: Whole milk was fed for approximately 6 wk., followed by reconstituted skimmilk. This was replaced gradually with a concentrate mixture containing 33 lb. nonfat dry milk solids, 66 lb. degermed corn meal, 1 lb. NaCl, 500,000 units of vitamin A as vitamin A acetate, and 142,000 units of vitamin D as irradiated yeast. When the calves averaged 8 mo. of age, the concentrate mixture was changed to 100 lb. degermed corn meal, 3 lb. crystal urea, 1 lb. NaCl, 500,000 units of vitamin A, and 142,000 units of vitamin D. Both of these mixtures contained approximately 16% crude protein equivalent. Cobalt was fed to all animals throughout the experiment, at the rate of 12.5 mg. twice a week as a drench.

The experimental animals were divided into four replications containing four animals each. Within each replication, one animal was fed 156 mg. $CuSO_4 \cdot 5H_2O$ per 100 lb. body weight twice a week. Another received 397 mg. Fe from carbonyl iron powder per 100 lb. body weight twice a week. A third animal received the same amounts of both of these elements, and the fourth animal was kept as a control. These amounts of iron and copper were approximately those reported by Knoop *et al. (2)* as meeting the requirements of dairy calves. Mineral supplementation was started when the calves averaged 4 mo. of age. Gelatin capsules were used as the method of feeding. After 2 mo., the rates of iron and copper fed were increased to four times the original levels. This change was made because the anemic condition of the calves had not been corrected, and it was felt that relatively large amounts of both iron and copper should be fed to make sure that the intake of these elements was adequate. Hay and basal concentrate mixture were fed ad libitum to the calf with the lowest feed consumption in each replication. The other animals in each replication

were given the amounts of feed consumed by that animal. As the experiment progressed, it became impossible to keep hay and concentrate consumption in balance. Beginning when the animals averaged 12 mo. of age, the hay was ground and mixed with the concentrate in a 2 to 1 ratio.

By the time the experimental animals had reached 14 mo. of age, it had become obvious that no response to iron or copper could be expected. It was decided at this time to try other treatments superimposed upon the iron and copper supplementation, and to allow ad libitum feeding. At this time, the members of one replication were injected with radioactive iodine and then slaughtered, to measure thyroid functioning. Immediately after this, iodine was fed to the members of one of the remaining replications at the rate of 5.25 mg. KI twice a week as a drench. Zinc at the rate of 302 mg. $ZnSO_4 \cdot 7H_2O$ per 100 lb. body weight was added to the daily ration of this same group. A second replication was fed this amount of zinc without iodine, and the members of the third replication were kept as controls. After a period of 3 mo., 25% of the concentrate mixture of those animals getting both iron and copper and those getting neither was replaced with dehydrated alfalfa pellets purchased in Kansas, and the previous treatments were continued. This was done to see if the growth-promoting properties of alfalfa, as evidenced in the experiment carried out with rabbits, could be observed. These treatments were continued for a period of 2.5 mo., until all of the animals were slaughtered and their thyroids removed and weighed.

Rabbit studies. The timothy hay used in these studies was harvested in 1953 from the same fields as the hay used in the calf studies. It was ground in a hammer mill to pass a ⅜-inch screen. The composition of the basal diet in percentages was, ground timothy, 75; wheat gluten, 10; hydrogenated vegetable oil, 7.9; dextrose, 6; NaCl, 1, and A and D percomorph oil, 0.1. All supplements to this diet were made at the expense of the dextrose. A diet in which ground alfalfa hay replaced the timothy of the basal diet served as a control.

Dutch belted rabbits 4 wk. of age were selected from a group weaned at 3 wk. of age and that had had access to the basal diet only from the age of 2 wk. The rabbits were individually caged and fed ad libitum. Double-demineralized water was offered the animals throughout the experiment. A randomized block design with six replications was used with rabbits, which were randomized to the treatments from weight-sex groups.

In one experiment, treatments included the following supplements to the basal diet: alfalfa ash (0.4 g. per 100 g. diet), cation and anion fractions obtained from an equivalent amount of alfalfa, and potassium iodide (25 μg. per 100 g. diet). In another experiment, the basal diet was supplemented with 5% alfalfa (second cutting, 1954 crop from Ithaca, New York).

EXPERIMENTAL RESULTS WITH CALVES

Composition of feeds. The chemical composition of the feeds included in the basal rations fed the calves is shown (Table 1). These rations were low in

iron, copper, cobalt, iodine, and magnesium. Zinc was relatively low, but a requirement has not been established. Calcium and phosphorus intakes were low before the addition of dicalcium phosphate, and particularly low following the change to urea. Magnesium was not added to the ration because deficiency levels were not found in the blood of these animals or others fed similar rations.

TABLE 1
Composition of feeds

	Timothy hay	Degermed corn meal	Nonfat dry milk solids[a]
Protein *(%)*	4.24	8.06	33.5
Fat *(%)*	1.74	0.36	0.9
Fiber *(%)*	32.97	0.32	0.2
Ash *(%)*	3.49	0.26	7.6
H_2O *(%)*	8.32	10.98	6.1
Ca *(%)*	0.25	0.03	1.26
P *(%)*	0.13	0.04	1.03
Fe *(p.p.m.)*	36.7	4.5	52.0
Cu *(p.p.m.)*	2.6	1.3	11.5
Mn *(p.p.m.)*	56.7	1.5	2.2
Co *(p.p.m.)*	0.03	0.02	0.11
Zn*(p.p.m.)*	17.8	2.9	—
I *(p.p.b.)*	35.0	34.0	—
K *(%)*	1.39	0.12	1.67
Mg *(%)*	0.07	0.01	0.11

[a] Values taken and calculated from *Composition of Concentrate By-Products Feeding Stuffs.* National Academy of Sciences. National Research Council Publ. 449. June, 1956.

Feed consumption and growth. Average body weights and TDN consumption for the heifers in the various treatments are shown (Figures 1 and 2, respectively). It can be seen that the animals in all treatments grew at a moderate but subnormal rate until the nonfat dry milk solids were removed from the concentrate mixture and urea was added. This change resulted in a rather marked decrease in feed consumption and growth rate. When the animals were changed to the ground hay–concentrate mixture, nutrient intake was depressed still further and the animals lost weight. In general, the rate of growth was simply a reflection of nutrient intake expressed as calculated TDN. All evidence from this and related studies indicates that feed consumption was low because of the lack of an appetite-stimulating factor, not because of the feed being particularly unpalatable. It appears that this factor was most deficient in the ration containing urea.

During the entire period of the experiment, there was no indication that either copper or iron alone, or together, stimulated appetite or increased body weight gains even at levels of approximately four times the published requirements. This does not mean necessarily that iron and copper were not deficient but that a deficiency of some other factor may have prevented a response.

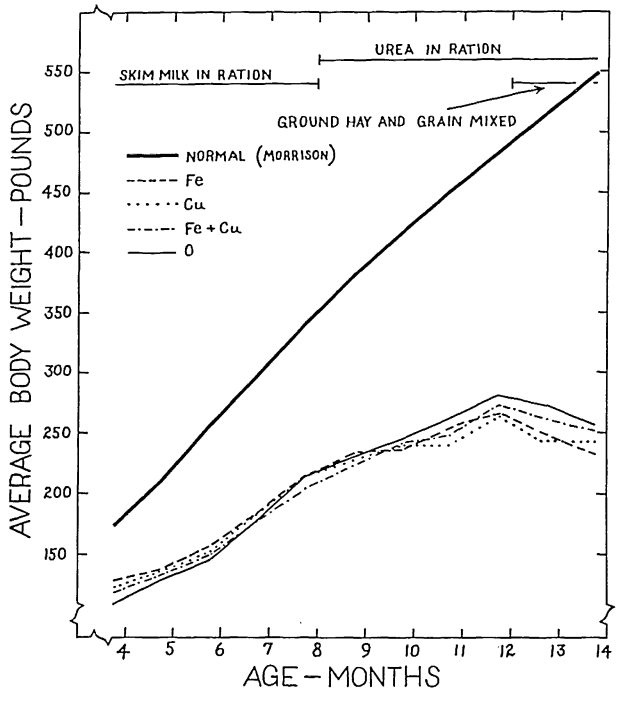


FIG. 1. Average body weights of calves fed various different mineral supplements.

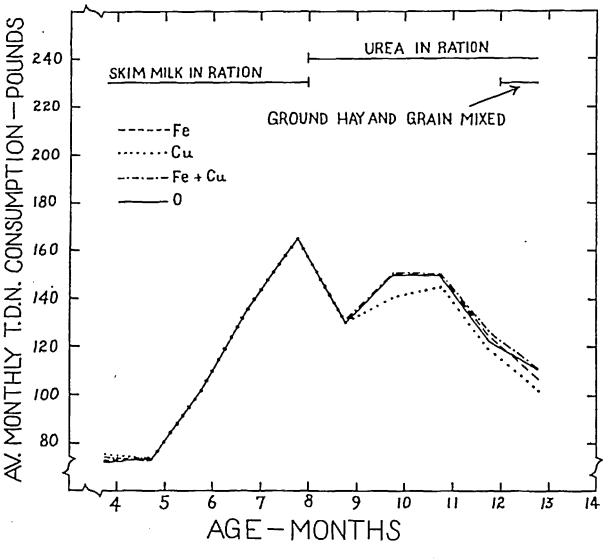


FIG. 2. Average total digestible nutrient consumption of calves fed different mineral supplements.

Inorganic phosphorus of blood. After the experiment got under way, it was observed that serum inorganic phosphorus dropped more rapidly in the control animals than in those getting either iron or copper, or both. These results are shown (Figure 3). Differences between the controls and those fed copper alone, or copper and iron, were significant at the 5% level. Differences between animals fed iron and copper, or iron alone, were significant at the 5% level.

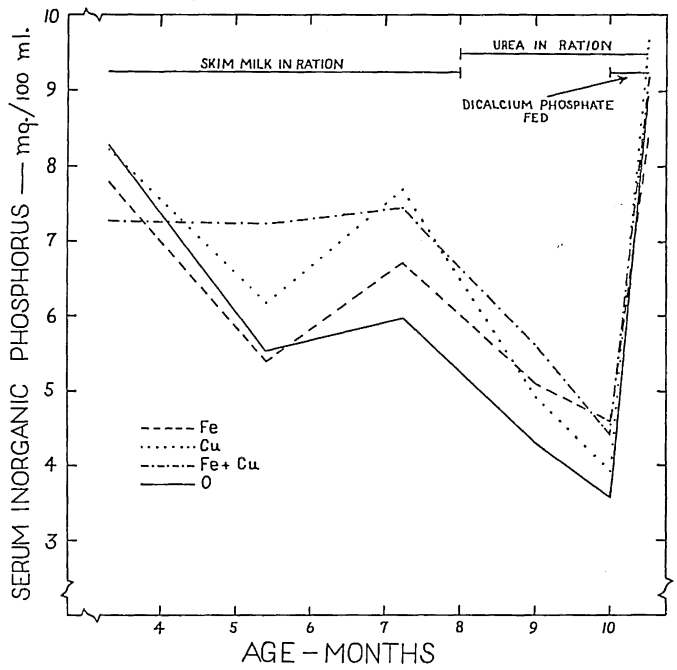


FIG. 3. Average serum inorganic phosphorus levels of calves fed different mineral supplements.

After the removal of nonfat dry milk solids from the ration, phosphorus deficiency developed so quickly that one animal was injured in the rear quarters and had to be removed from the experiment. After this happened, all animals were fed 40 g. of dicalcium phosphate per day, and differences due to iron or copper supplementation disappeared and serum inorganic phosphorus levels returned to normal. These results suggest that under conditions of low phosphorus intake, both iron and copper may influence the level of inorganic phosphorus in the blood, and the combination appears to exert a greater influence that either one alone. However, supplementation of the ration with phosphorus did not stimulate growth or feed consumption.

Hemoglobin levels. Hemoglobin levels were somewhat low throughout the experiment, with average values falling between 8 and 10 g. per 100 ml. of blood. There was no evidence that the feeding of iron, copper, or both, had any significant effect on hemoglobin level at any time during the experiment.

Supplementary studies. Results from the injection of radioactive iodine, which are being published elsewhere, showed that the uptake and conversion of this element were many times greater than normal. The feeding of zinc, or iodine and zinc, did not increase feed consumption or body weight above the controls. Subsequent studies with radioactive iodine showed that the feeding of iodine for a period of 2 mo. had corrected the abnormal thyroid functioning, although no other response could be observed. When the remaining animals were slaughtered at the end of the experiment, thyroid weights were found to be as given (Table 2). These data show a highly significant difference in thyroid weight between those animals which received iodine and those which did not. It appears that the increase in thyroid weight of the animals not fed iodine took place after the first animals were slaughtered. Copper supplementation appeared to increase thyroid size slightly (not significant at 5% level), but iron, zinc, and alfalfa had no observable effect on thyroid size.

TABLE 2
Thyroid weights of calves

Replication No.	Animal No.	Supplement: Beginning at 4 mo.	Supplement: Added at 14.5 mo.	Age when slaughtered	Thyroid weight
				(mo.)	*(g.)*
3	135	Cu		14	14
	136	Fe + Cu		14	13
	137	O		14	10
	138	Fe		14	6
	Av.				10.7
1	127[a]	Fe + Cu	I + Zn	20	13
	128	Fe	I + Zn	20	10
	129	Cu	I + Zn	20	15
	130[a]	O	I + Zn	20	17
	Av.				13.7
2	131[a]	Fe + Cu		20	52
	132[a]	O		20	36
	133	Cu		20	—
	134	Fe		20	46
	Av.				44.7
4	139[a]	O	Zn	20	19
	140	Fe	Zn	20	—
	141	Cu	Zn	20	44
	142[a]	Fe + Cu	Zn	20	35
	Av.				32.7

All Fe alone	20.7	All Cu (incl. Fe + Cu)	26.6
All Cu alone	24.3	All no Cu	20.6
All Fe + Cu	28.2	All alfalfa	28.7
Controls	20.5	No alfalfa	28.7

[a] Received Kansas alfalfa after 17½ mo. of age.

The feeding of the alfalfa pellets seemed to result in considerable appetite and growth response in some animals, but little or no growth in others. This inconsistent response pattern might be explained on the basis of varying pituitary changes, comparable to those found in animals maintained on a similar feeding program. Such changes involving the tissues which produce growth hormone might be corrected only slowly, if at all. It seems, therefore, that further studies will have to be carried out in order to understand this problem.

EXPERIMENTAL RESULTS WITH RABBITS

Poor growth, mild anemia, and hyperplastic thyroids also were evidenced in the rabbits fed the heavily fertilized timothy hay (shown in Table 3). The hyperplasia of the thyroid gland was reduced by the feeding of alfalfa ash or iodine supplements, but not by the cation or anion fractions of the ash. Iodine analysis indicated that 100 g. of the basal diet contained approximately 10 γ of iodine, that the alfalfa ash supplement contributed 2 γ, and that the iodine was lost in the preparation of the cation or anion fractions. Because iodine supplements alone reduced the size of the thyroid gland, it would appear that the iodine content of the basal diet furnished less than the rabbit's requirement or that the availability of the timothy iodine was low.

TABLE 3

Studies of the timothy hay with rabbits

Treatment	Weight gain	Hb per 100 ml. blood	Thyroid per 100 g. body weight
	(g.)	*(g.)*	*(mg.)*
	Experiment 1		
Basal	564	9.9	11.4
+ Alfalfa ash [a]	593	9.5	8.4
+ Cations [a]	510	10.2	13.3
+ Anions [a]	589	9.5	12.3
+ Iodine [b]	528	8.9	7.4
Control [c]	1,103	12.0	6.9
	Experiment 2		
Basal [a]	695	10.8	
Alfalfa [a]	965	12.8	

[a] At a level of 5% alfalfa, or its equivalent in ash (500° C. for 16 hr.). Added at the expense of dextrose in basal diet.

[b] 26 μg. per 100 g. diet.

[c] Alfalfa replacing timothy in basal diet.

The supplementation of the basal diet with 5% alfalfa improved body weight gain and hemoglobin formation in the rabbits; the ash of a comparable amount of alfalfa had no effect on these variables. This result suggests that alfalfa contains an organic factor or a volatile mineral other than iodine which is essential to the adequate nutrition of the rabbit. That alfalfa contains such a nutriment has been suggested by Thacker and Ellis *(3)*.

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ANAEROBIC PURINE DISSIMILATION BY WASHED SUSPENSIONS OF BOVINE RUMEN BACTERIA[1,2]

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SUMMARY

Xanthine, uric acid, and guanine hydrochloride were degraded completely, at a slow rate, by WCS of bovine rumen bacteria. The end-products formed were carbon dioxide, ammonia, and acetic acid, and the fermentation pattern was similar to that observed in clostridial-type organisms. Hypoxanthine was degraded incompletely, whereas adenine was neither decarboxylated nor deaminated under the conditions employed. Gases other than carbon dioxide (hydrogen and/or methane) never were encountered as end-products of purine degradation and neither were lactic, formic, propionic, and butyric acids. With the exception of hypoxanthine, WCS deaminated purines to a greater extent under alkaline than under acidic conditions. In experiments dealing with xanthine decarboxylation, less carbon dioxide was formed from this substrate than that observed for the corresponding endogenous value. This was not true of other purines. Kinetic treatment of the data suggested that a carbon dioxide utilizing or fixing mechanism was involved in xanthine dissimilation. Purine interconversions could not be demonstrated by paper chromatography. Differential spectrophotometric methods showed that xanthine and uric acid were degraded completely, hypoxanthine was partially degraded, and adenine was not dissimilated.

Purines are known constituents of plants and they exist in the free state or bound as nucleosides or nucleotides. Ferguson and Terry *(11)* reported the presence of purines in grassland herbage, i.e., adenine, guanine, xanthine, and hypoxanthine. The nonprotein nitrogen content of grassland herbage represents from 15 to 20% of the total nitrogen present, and from 50 to 60% of the nonprotein nitrogen fraction is ammonia, nitrate, acid amides, and amino acids. The remainder consists of purines, possible pyrimidines, either free or bound *(11)*. Guanine and adenine, well-known constituents of nucleic acids, have been found in the free state in alfalfa leaves *(28)*. Hypoxanthine, xanthine, and uric acid occur primarily in the free state, and uric acid has been observed in leaves and seeds in concentrations of 250 mg. per kilogram *(12)*. Therefore, free or bound purines are, in fact, ingested in feeds given to ruminants. Experiments were undertaken to determine to what extent catabolic activity was exhibited by washed cell suspensions (WCS) of bovine rumen bacteria on this group of compounds.

EXPERIMENTAL PROCEDURE

Rumen samples were obtained from a 4-year-old fistulated Holstein cow approximately four hours after morning feeding. The animal's diet consisted of U. S. No. 2 leafy alfalfa hay, ad libitum, supplemented daily with 16% protein concentrate. Methodology employed in processing and standardizing the WCS

Received for publication June 13, 1957.

[1] Hereafter, WCS designates washed cell suspensions of bovine rumen bacteria.

[2] Scientific Article No. A632, Contribution No. 2814 of the Maryland Agricultural Experiment Station.

has been described previously by Doetsch *et al. (10)*. Total nitrogen determinations on WCS indicate that 1.0 ml. of the suspension was equivalent to 1.3 ±0.2 mg. nitrogen. These cells were used for both manometric studies and long-term dissimilation reactions, described below.

Buffer solutions containing 0.067 *M* Na_2HPO_4 and KH_2PO_4 were combined in appropriate amounts to give the desired pH *(24)* immediately before use, then boiled and rapidly cooled and sufficient $Na_2S \cdot 9H_2O$ added to give a final concentration of 0.02% (w/v). Stock purine solutions were prepared by dissolving 5 m*M* in 10 ml. *N* NaOH in a 100-ml. volumetric flask, made up to volume with distilled water. These stock solutions of 0.05 *M* purines were diluted further with distilled water to the concentration levels desired. Immediately before use they were neutralized with HCl, the pH being adjusted to the desired value. All solutions were stored at 4° C. and the pH was checked electrometrically.

All experiments were performed under anaerobic conditions, atmospheric oxygen was displaced by thoroughly flushing the system with N_2, He, or a mixture of 1% (v/v) CO_2 in N_2. All gases were freed from oxygen by passage over reduced copper filings at 600° C.

Long-term dissimilation tests were modified as follows: *(a)* 50-ml. Erlenmeyer flasks were used with 60 μM purine substrate in 9 ml. of 0.067 *M* phosphate buffer at the desired pH [containing 0.02% (w/v) $NaS \cdot 9H_2O$], to which were added WCS and *(b)* 50-ml. Erlenmeyer flasks with 200 μM substrate in 12 ml. phosphate buffer, to which 8 ml. of WCS were added. Flasks were incubated at 39° C. in a water bath for periods ranging from 12 to 72 hr. Endogenous controls always were included and after incubation suitable inhibitors (depending on the type of analysis to be performed) were added to aliquots set aside for biochemical assay.

Manometric techniques (short-term dissimilation studies) also were employed, and carbon dioxide evolution and gases other than carbon dioxide evolved were determined using the direct method *(24)* and a modified Dixon-Keilin technique. In all instances, the fluid volume was 3.0 ml. and 0.5 ml. of purine (varying concentrations), and 0.2 ml. of 10 *N* H_2SO_4 containing 5% (w/v) perchloric acid were placed in the side-arms. Carbon dioxide was absorbed by 10% (w/v) KOH in the center well. Acid production also was demonstrated manometrically using bicarbonate-phosphate buffer under an atmosphere of 1% (v/v) CO_2 in N_2.

Dixon-Keilin flasks with two side-arms were employed with conventional Warburg constant-volume respirometers. Substrate (varying concentrations) and the acid-inhibitor were placed in the side-arms and KOH was placed in the stopcock opening which was exposed to the atmosphere of the reacting system. At the start, acid-inhibitor was tipped into one of the duplicate flasks giving initial "bound" carbon dioxide. After release of all bound carbon dioxide, the stopcock was turned, exposing the alkali to the atmosphere within the flask, thereby absorbing all carbon dioxide in the gaseous phase. This caused a drop in the manometer reading. The reaction was continued in the other

flask (duplicate) in the presence of carbon dioxide, causing a manometric change. At the end of a measured interval, acid-inhibitor was tipped into the second flask, which was similarly exposed to alkali. The total volume of carbon dioxide in each flask was estimated quantitatively. By use of this method, the following could be determined: initial and final bound carbon dioxide, initial and final total carbon dioxide, and final gases other than carbon dioxide (assuming the initial flask to have a value of zero). Reactions could be carried out in the presence of carbon dioxide within definite limits.

Lactic acid was determined colorimetrically *(2)*, and volatile fatty acids were determined by partition chromatography *(17)*. Known concentrations of volatile acids were used as markers with each series of fatty acid determinations, to verify identifications and quantitative estimations. Ammonia was estimated quantitatively by microdiffusion analysis *(9)*, as modified by Tonhazy *(23)*, as well as by direct titration with 0.02 *N* HCl. In the latter case, ammonia was evolved from an alkaline (pH 10) deproteinized (trichloroacetic acid, final concentration 10% w/v) aliquot by steam distillation (Pregl apparatus) and absorbed in 4% (w/v) boric acid solution containing a mixed sensitive indicator *(22)*. Ammonia standards were treated in the same manner, and in determinations on purine dissimilation, corrections for the endogenous were made by substraction. Total nitrogen was determined using a micro-Kjeldahl procedure *(18, 24)*. Protein nitrogen was determined indirectly as the difference between total nitrogen and nonprotein nitrogen. Deproteinization was carried out using trichloracetic acid [10% (w/v), final concentration] or perchloric acid [5% (w/v), final concentration].

Residual purine determinations were measured by following changes in ultraviolet absorption curves of deproteinized incubation mixtures at 260 and 280 mμ *(6, 25)*. Paper chromatography studies were carried out on purine interconversions, using one-dimensional chromatograms (Whatman No. 1 paper, sealed glass cylinder). These studies were performed at room temperature for 24 hr., using 77% (v/v) n-butanol, 10% (v/v) formic acid in distilled water *(19)*. Purine spots were identified by inspection under a "Mineralight" lamp possessing an ultraviolet filter emitting at approximately 250 mμ.

RESULTS

Deamination of xanthine, uric acid, and guanine hydrochloride occurred at pH 6.9 (as illustrated in Table 1). These purines were deaminated under neutral and alkaline conditions, and insignificant ammonia production was observed with hypoxanthine and adenine (under the same conditions). Excepting hypoxanthine, less ammonia was liberated from purines under acid conditions.

Studies on fatty acid production from xanthine are presented (Table 2). Acetic acid was the major volatile fatty acid end-product encountered. If assumed that when one mole of xanthine is degraded, one mole of acetic acid is formed, the results in Table 2 indicate that 90% of the xanthine was dissimilated. Volatile fatty acid production from other purines is summarized

TABLE 1

Anaerobic deamination of purines by rumen WCS[a]

Substrate	Initial ammonia	Final ammonia	Final minus initial	Corrected for endogenous	Theoretical ammonia	Theoretical ammonia
(0.01 M)	*(μM)*	*(μM)*	*(μM)*	*(μM)*	*(μM)*	*(%)*
Hypoxanthine	2.6[b]	24.2[c]	21.6	16.2	40	40
Xanthine	3.6	49.4	45.8	40.4	40	101
Uric Acid	3.6	46.2	42.6	37.2	40	93
Guanine HCl	2.4	51.4	49.0	43.6	50	87
Adenine	2.4	14.1	11.7	6.3	50	13
Endogenous	2.4	7.8	5.4	....	....	....

[a] 200 μM substrate, total volume 20 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM NH_3/ml initially present.
[c] Values represent μM NH_3/ml after 48 hr. incubation.

(Table 3). The greatest difference was noted with xanthine (425 μM of acetic acid was produced). This represents 103% dissimilation, assuming the formation of one mole of acetate from one mole of xanthine. Small increases in propionic and butyric acids were observed in this instance with xanthine. Negligible amounts of fatty acids were formed from hypoxanthine and adenine; whereas, small but definite fatty acid production was noted from uric acid and guanine hydrochloride. Formic acid never was observed in experiments on purine degradation.

Lactic acid never was observed as an end-product in purine degradation studies, regardless of the time of assay (from 3 to 72 hr.). There was no increase in protein nitrogen during purine dissimilation by WCS during long-term studies. If growth were a complicating factor, results would be difficult, if not impossible, to evaluate.

Paper chromatography studies yielded essentially negative results on purine interconversions. Rf values compared favorably with those reported by Markham and Smith *(19)*. The conversion of hypoxanthine, uric acid, and guanine hydrochloride to xanthine was not observed using this method.

Residual purine concentration was estimated quantitatively by differential spectrophotometry (as shown in Table 4). The per cent theoretical purine given in this table compares favorably with the per cent theoretical ammonia (shown in Table 1). A slight discrepancy existed with xanthine, because dif-

TABLE 2

Volatile fatty acid formation from xanthine by WCS[a]

Volatile acids	0.005 *M* xanthine	Endogenous	Difference
	(μM)	*(μM)*	*(μM)*
Valeric and higher	10.1[b]	12.7	....
Butyric	48.3	46.5	1.8
Propionic	67.0	64.3	2.7
Acetic	245.0	191.0	54.0
Formic	0.0	0.0	0.0

[a] 60 μM xanthine, total volume 12 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM volatile fatty acids per 12 ml. fermentation liquor.

TABLE 3

Volatile fatty acid formation from purines by WCS[a]

Substrate	Valeric and higher	Butyric	Propionic	Acetic
(0.01 M)	*(μM)*	*(μM)*	*(μM)*	*(μM)*
Hypoxanthine	Less than 6.0[b]	21.8	30.6	225
Xanthine	Less than 6.0	28.1	112.4	425
Uric Acid	Less than 6.0	25.0	96.8	263
Guanine HCl	Less than 6.0	18.8	100.0	258
Adenine	Less than 6.0	15.6	93.7	212
Endogenous	Less than 6.0	18.8	93.7	219

[a] 200 μM purines, total volume 20 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM volatile fatty acid per 20 ml. fermentation liquor.

ferential spectrophotometry gave a value of 92%; whereas, that obtained from ammonia and acetic acid formation was 101 and 103%, respectively.

Carbon dioxide production from purines was estimated manometrically and is shown (Figure 1 and Table 5). These short-term experiments indicated carbon dioxide evolution from uric acid, guanine hydrochloride, and hypoxanthine. Adenine was not decarboxylated by WCS. A definite decrease in carbon dioxide evolution consistently was observed with xanthine. Less carbon dioxide was formed from WCS plus xanthine, than from WCS alone. Because xanthine was degraded readily in long-term dissimilation studies as determined by ammonia production, volatile fatty acid formation, and disappearance of substrate (differential spectrophotometry), this phenomenon of decreased carbon dioxide evolution suggests a utilizing or fixing mechanism. Gases other than carbon dioxide (hydrogen and/or methane) never were formed in amounts substantially greater than the endogenous, in manometric studies.

Carbon dioxide and ammonia were estimated quantitatively, using varying concentrations of xanthine (as illustrated in Figure 2). Results of this experiment definitely suggest the involvement of a carbon dioxide fixing or utilizing mechanism. As the xanthine concentration increased, there was a marked decrease in the apparent decarboxylation rate and an increase in the deamination

TABLE 4

Residual purine determination from dissimilation studies with WCS[a]

Substrate	Incubation time	Optical density reading at: 250 mμ	260 mμ	280 mμ	Difference (260–250) or 260–280)	Residual purine	Theoretical purine
	(hr.)					*(μM/ml)*	*(%)*
Hypoxanthine	0	.....	0.648[b,c]	0.024	0.424	10.0	....
	48	.....	0.392	0.014	0.378	6.1	40
Xanthine	0	0.444	0.657	.....	0.213	10.0	....
	48	0.026	0.043	.....	0.017	0.8	92
Uric Acid	0	.....	0.040	0.135	0.095	10.0	....
	48	.....	0.067	0.058	0.009	0.9	90
Adenine	0	.....	1.208	0.459	0.749	10.0	....
	48	.....	0.927	0.280	0.647	8.6	14

[a] 200 μM substrate, total volume 20 ml., incubated at 39° C. under 100% N_2.
[b] Optical density of deproteinized aliquots diluted 1/100 with 0.1 *N* H_2SO_4.
[c] Optical density substracted for endogenous.

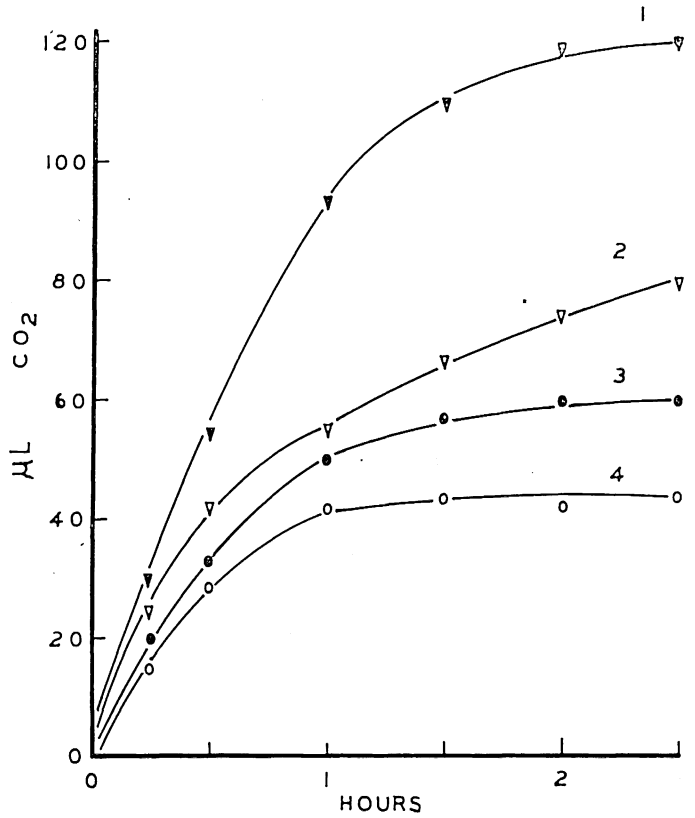


FIG. 1. Carbon dioxide evolution (determined manometrically) from 0.004 *M* purines: incubation 2.5 hr. at 39° C. under 100% N_2:1, uric acid; 2, hypoxanthine; 3, endogenous; 4, xanthine.

rate. In addition, acid production from xanthine (Figure 3) was substantially greater than the corresponding endogenous value. To determine whether carbon dioxide influenced xanthine dissimilation, a modified Dixon-Keilin technique was employed. As observed from results (Figure 4), diminished carbon

TABLE 5

Manometric decarboxylation of purines by WCS [a]

Substrate	Initial carbon dioxide	Final carbon dioxide	Final minus initial	Corrected for endogenous
(0.0033 M.)	*(μl.)*	*(μl.)*	*(μl.)*	*(μl.)*
Hypoxanthine	75[b]	150[c]	75	36
Xanthine	70	90	20	—19
Uric Acid	120	220	100	61
Guanine HCl	68	159	91	52
Adenine	65	107	42	3
Endogenous	61	100	39	....

[a] 10 μM substrate, total volume 3.0 ml., incubated 3 hr. at 30° C. under 100% N_2.
[b] Values represent μl. CO_2 initially present.
[c] Values represent μl. CO_2 formed after 3 hr. of incubation.

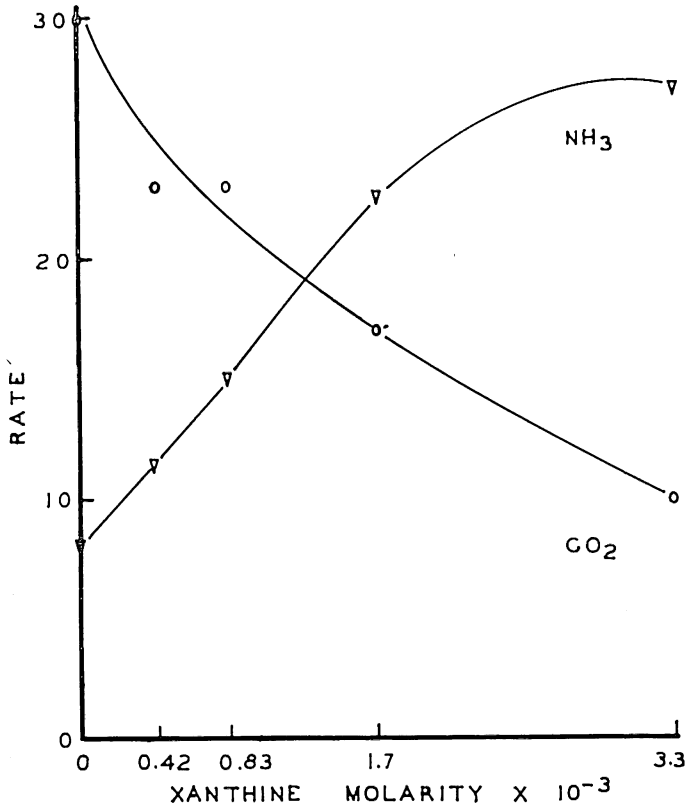


FIG. 2. Decarboxylation and deamination rates (expressed as μl. gases evolved/hr/mg N) from xanthine at various substrate levels (expressed as moles/liter $\times 10^{-3}$): incubation 3 hr. at 39° C. under 100% He.

dioxide evolution is obtained from xanthine in comparison to the endogenous. The fact that both methods (direct method *vs.* modified Dixon-Keilin) gave similar results indicates that sufficient bound carbon dioxide was present in the reacting system of the alkali-containing flask (direct method) to give reaction rates equivalent to those of the duplicate flask (without alkali).

Manometric decarboxylation rates were determined at various bacterial densities in the presence and absence of xanthine (Figure 4). Less carbon dioxide always was formed in the presence of this purine when compared to its corresponding endogenous value, at all bacterial densities. Since the initial carbon dioxide concentration increased directly with bacterial density (bacterial nitrogen content), the decarboxylation rate per mg. nitrogen of each point (Figure 4) was calculated and then plotted as a function of initial carbon dioxide concentration (Figure 5). From this graph, the endogenous decarboxylation rate decreases in a linear fashion as the carbon dioxide concentration increases. The reverse was true for the reaction observed in the presence of xanthine, because the decarboxylation rate is low at reduced carbon dioxide concentrations. However, the xanthine decarboxylation rate sharply

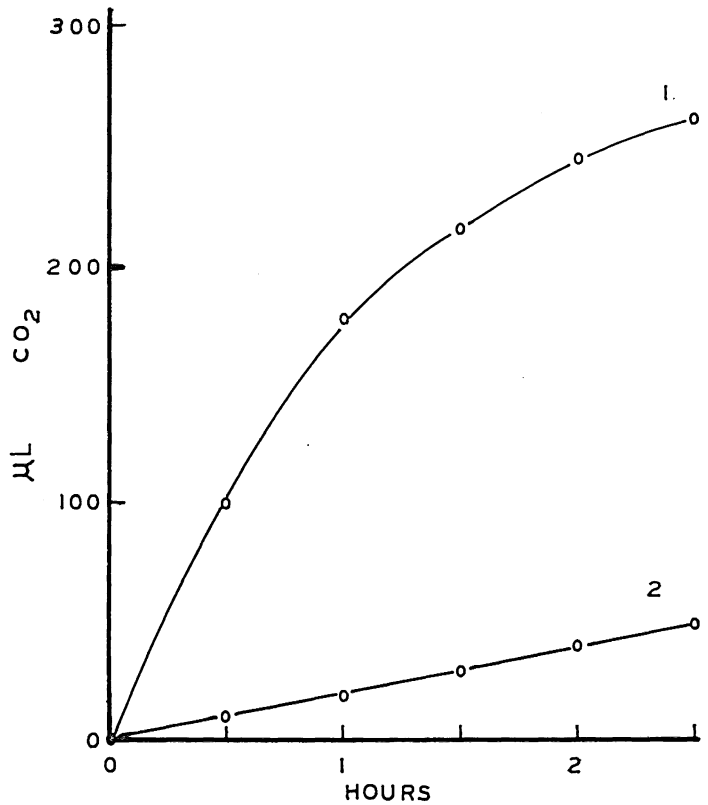


FIG. 3. Acid production (expressed as μl. CO_2 evolved) from 0.0017 *M* xanthine in 0.0015 *M* bicarbonate buffer: incubation 2.5 hr. at 39° C. under 1% CO_2 and 99% N_2: initial pH 7.1 (calculated): 1, xanthine; 2, endogenous.

increases, and approaches the endogenous value as the carbon dioxide concentration increases. In this same experiment, the production of hydrogen and/or methane was proportional to bacterial density (nitrogen) with both xanthine and WCS, and WCS alone (Figure 6). Slightly more hydrogen and/or methane was formed from this purine than from the endogenous, the rates being almost equivalent. This was not the case with carbon dioxide evolution. A direct proportionality seemed to exist between the formation of gases other than carbon dioxide and the amount of intracellular polysaccharide (ISS) present *(13)*. More hydrogen and/or methane were formed from cells containing larger quantities of ISS (determined qualitatively by iodine staining).

DISCUSSION

Theoretically, purines may have two possible fates in the rumen. They may be dissimilated by the rumen microflora and thereby utilized as a carbon or nitrogen source; or they may serve as growth factors for rumen bacteria. Studies employing the artificial rumen indicate that uric acid and allantoin are suitable nitrogen sources for the rumen microflora *(4)*. Other in vitro studies with mixed suspensions of rumen bacteria suggest that cellulose digestion im-

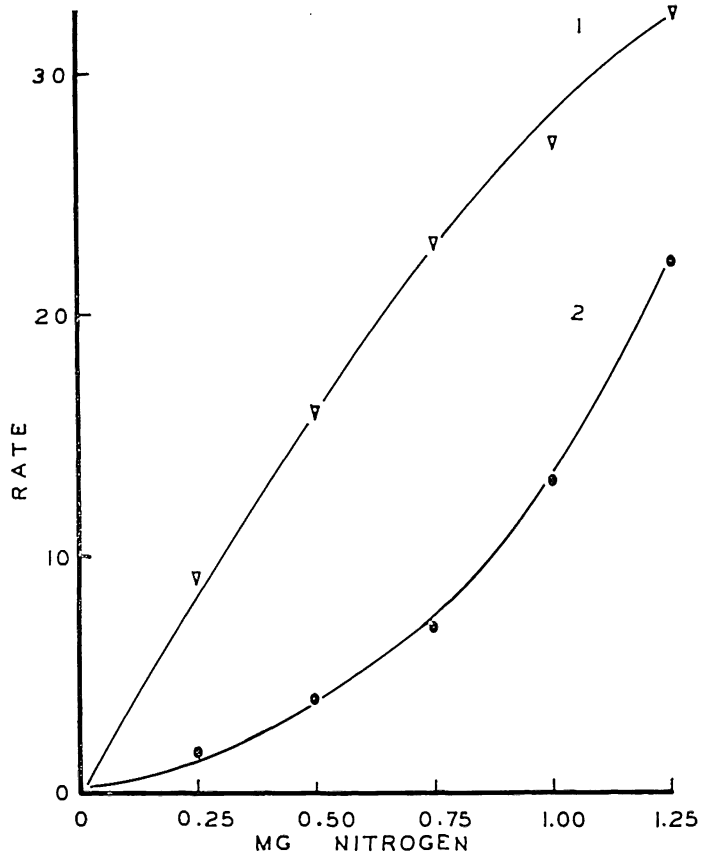


FIG. 4. Decarboxylation rates (expressed as μl. CO_2 evolved/hr.) from 0.0017 *M* xanthine at various bacterial densities (expressed as mg. *N*): incubation 2 hr. at 39° C. under 100% He : 1, endogenous; 2, xanthine.

proves considerably when purines are added to the reaction mixture *(5)*. In growth factor studies on the cellulolytic rumen bacterium, *Bacteriodes succinogenes,* purines and pyrimidines were used routinely in trace amounts, but the requirements of rumen microorganisms for these compounds have yet to be demonstrated *(7)*.

There are two general schemes for the anaerobic breakdown of purines *(21)*. Either the imidazole ring is split, yielding pyrimidine derivatives as intermediates as illustrated by *Micrococcus aerogenes (26),* or the pyrimidine ring is split, yielding imidazole derivatives, i.e., as with *Clostridium acidiurici* and *Clostridium cylindrosporum.* In case of degradation by clostridia, the major end-products are ammonia, carbon dioxide, and acetic acid; whereas, with *M. aerogenes* these same end-products are encountered in addition to hydrogen, propionic and lactic acids, with trace amounts of pyrimidines. WCS of bovine rumen bacteria dissimilate purines, in vitro, using the same fermentative pattern observed with the nonrumen clostridial types. Other similarities are that both degrade xanthine, uric acid, and guanine hydrochloride completely, with

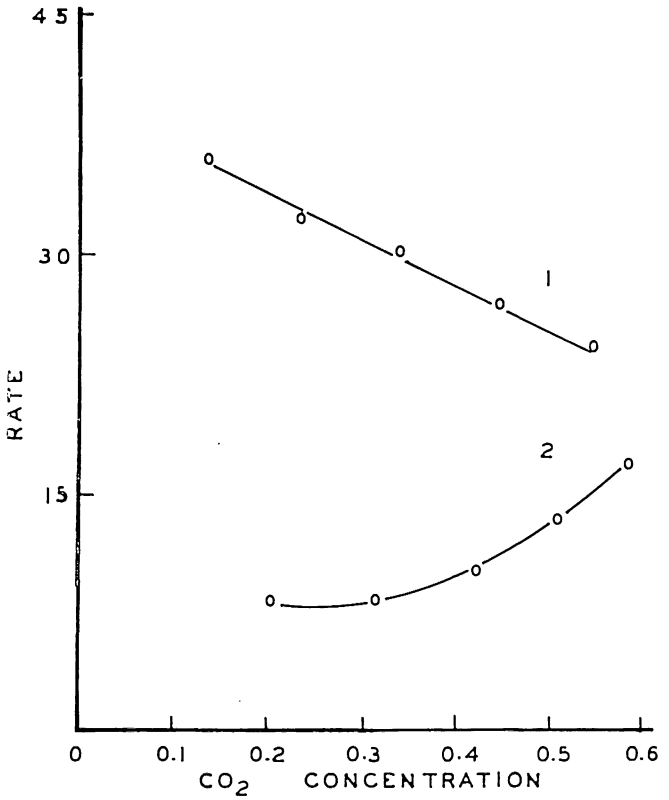


FIG. 5. Decarboxylation rates (expressed as μl. CO_2 evolved/hr/mg N) plotted as a function of CO_2 concentration (expressed as per cent initial CO_2 present per total volme): incubation 2 hr. at 39° C. under 100% He : 1, endogenous; 2, xanthine.

the formation of ammonia, carbon dioxide, and acetic acid; degrade hypoxanthine incompletely, and do not degrade adenine. This appears unusual, as it is known that anaerobic micrococci exist in large numbers *(15)* in the rumen, whereas clostridia rarely are encountered. If the micrococcal fermentation pattern was exhibited by WCS, then lactic, propionic acids, hydrogen, and/or methane would have been detected at some interval as end-products during in vitro dissimilation studies.

Purines are dissimilated by WCS at a slow rate, and deamination apparently proceeds after a prolonged incubation period, from 5 to 18 hr. This suggests that substrates as insoluble as purines require a prolonged reaction interval before significant dissimilation can be detected by biochemical assay of end-products. In this respect, in vitro dissimilation of purines may be compared to cellulose degradation *(8)*, as both are degraded by WCS after an incubation period of at least 24 hr. This further suggests that the rate of decomposition of any substrate is not indicative of its potential nutritive value in the rumen, because in most instances cellulose (insoluble carbohydrate) is the primary carbon source for ruminants. By analogy, the slow rate of attack on purines by WCS may not indicate its potential nutritional value. Deamination under

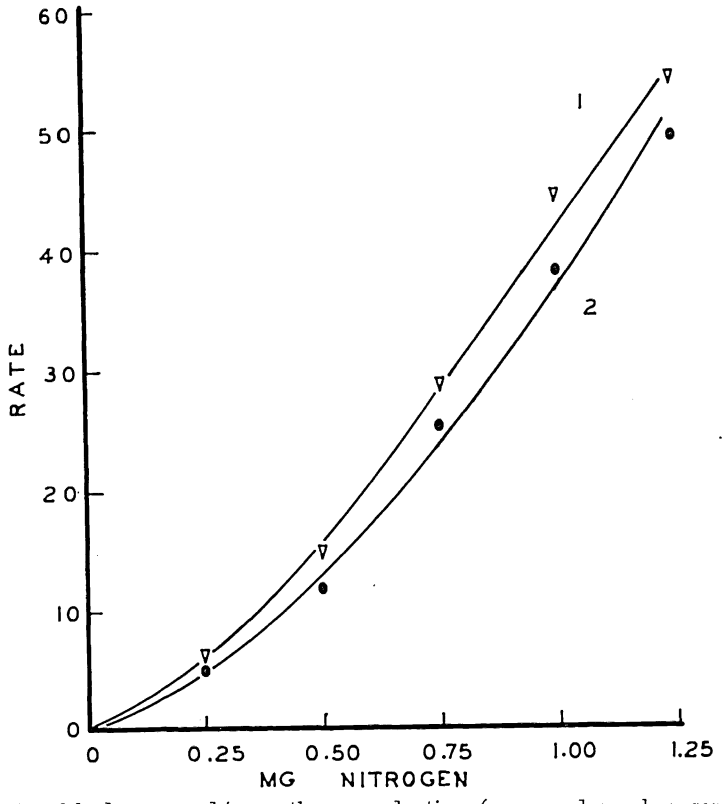


FIG. 6. Rate of hydrogen and/or methane production (expressed as μl. gases evolved/hr.) from 0.0017 *M* xanthine at various bacterial densities (expressed as mg. *N*): incubation 2 hr. at 39° C. under 100% He:1, xanthine; 2, endogenous.

alkaline conditions was greater than or equivalent to that observed at neutral pH values, excepting hypoxanthine. Increased solubility of purines at higher pH values may be a factor responsible for the increased deamination rate.

The activity of WCS on xanthine (as estimated by ammonia liberation, volatile fatty acid production, and residual purine determination) always was greater than that observed for guanine hydrochloride and uric acid. This would be expected if xanthine is the common intermediate in the degradation of all purines *(3)*. Constitutive enzymes are responsible for xanthine degradation, and adaptive enzymes are involved in the breakdown of uric acid and hypoxanthine. The inability to form adaptive enzymes may be due to the absence of these purines in the rumen or the inactivation of these purine-attacking enzymes during differential centrifugation procedures.

An indirect approach was used to determine whether carbon dioxide influenced reaction rates involved in xanthine degradation. Reaction rates were determined at various bacterial densities, using one substrate concentration level. In decreasing bacterial densities, carbon dioxide concentrations were decreased proportionally. (Carbon dioxide contamination from WCS is decreased at diminishing bacterial densities, until a critical carbon dioxide

concentration level is reached, at which point the reaction rate is not influenced.) Kinetic treatment of the data (Figures 4 and 5) indicates that the greatest differences in decarboxylation rates between xanthine and the endogenous is at minimal carbon dioxide concentrations, where the least amount of bacterial cells was present. At this level, the endogenous activity would be negligible and any carbon dioxide–utilizing response would be magnified. At high bacterial densities, endogenous decarboxylation reactions and contaminating bicarbonate of the mixed suspension are greater, and any carbon dioxide–utilizing response would be masked.

This type of effect was not observed with hydrogen and/or methane formation from the same series of experiments (Figure 6). The linear decrease observed in the endogenous decarboxylation rate at increasing carbon dioxide concentrations (Figure 5) suggests that a carbon dioxide–utilizing mechanism is normally present in WCS *(14)*. Carbon dioxide may act as the ultimate hydrogen acceptor forming methane, as demonstrated by McNeill *(20)* with WCS of rumen bacteria, or acetic acid as reported with *Clostridium aceticum (27)*. Carbon dioxide utilization during anaerobic purine dissimilation already has been established with clostridia *(1, 16)*. A similar finding is suggested in this study by indirect evidence: *(a)* xanthine is dissimilated readily in all long-term studies, yet carbon dioxide evolution from this purine always is smaller than the corresponding endogeneous value; *(b)* the xanthine decarboxylation rate decreases and the deamination rate increases as the xanthine concentration is increased (Figure 2); *(c)* acid production from xanthine always is greater than the corresponding endogenous value (Figure 3).

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TECHNICAL NOTES

EFFECT OF SODIUM CHLORIDE ON THE CURD TENSION OF RENNET-COAGULATED MILK

Among the soft pickled varieties of cheese which are usually made in warm climates, Domiati cheese is unique in being salted at the first stage of manufacture by adding it directly to the milk before renneting. The addition of an appreciable amount of salt to the milk, which sometimes reaches 15%, increases the time of coagulation and causes the formation of a softer curd *(3)*.

The study was undertaken, therefore, to determine quantitatively the effect of various concentrations of salt on the curd tension *(5)* of milk.

The investigations were carried out on cows' milk, both raw and pasteurized. In each case, the milk was warmed to 35° C. Samples of 100 ml. were placed in glass beakers 4.5 cm. in diameter and 6.5 cm. high. The beakers were then incubated in a water-bath regulated at 35 ± 0.5° C. One per cent of starter [either *Streptococcus faecalis (1)* or commercial *Streptococcus lactis*] was used in some of the pasteurized milk experiments. A ripening period of 1 hr. was allowed in this case. The required amount of salt was then dissolved in the milk. Standard Hansen's liquid rennet (diluted 1:20) was added at the rate of 1 ml. for each 10 lb. of milk. The rennet curd tension, after the appropriate time of coagulation, was measured by the curd-o-meter.[1]

The effects of rennet coagulation time and concentration of salt on the curd tension of raw milk are shown (Fig. 1). The curd tension is seen to increase with rennet coagulation time but to decrease with increasing salt concentration. It took 1 hr. and 45 min. for the milk containing 15% salt to reach the same curd tension value of 20 g. reached in 45 min. by the unsalted milk. In additional experiments with milk containing 15% salt, but not included in Fig. 1, it was found that the curd tension value had reached 85 g. at 3 hr. and 100 g. at 3 hr. and 30 min.

The addition of sodium chloride definitely reduced the curd tension of the milk for each coagulation period. Three possible explanations for this phenomenon have been presented by Fahmi and Sharara *(3)*. The reduction of the curd tension by the addition of other sodium salts has been reported by Tracy and Corbett *(9)*.

Figure 2 illustrates graphically the effect of salt and starters on the curd tension of ordinary pasteurized milk held 2 hr. and 15 min. for coagulation. Salt reduced the curd tension of pasteurized milk as it had for raw milk. The addition of 1% starter of the *S. faecalis*, and allowing 1 hr. ripening period before the addition of salt, definitely increased the firmness of the curd. In this case, the 2.5% salt did not reduce the curd tension; however, higher concentrations of salt did reduce the curd tension of milk. The addition of 1%

[1] Submarine Signal Co., Boston.

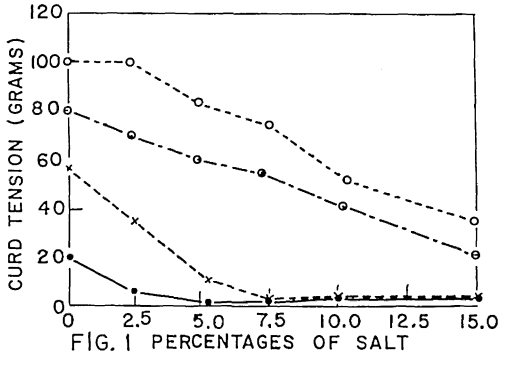


FIG. 1. Effects of salt and rennet coagulation period on the curd tension of raw milk.

Legend:

- ●———● Rennet coagulation period of 45 min.
- × - - - - × Rennet coagulation period of 1 hr. and 15 min.
- ⊙—·—·—⊙ Rennet coagulation period of 1 hr. and 45 min.
- ○ · · · · · · ○ Rennet coagulation period of 2 hr. and 15 min.

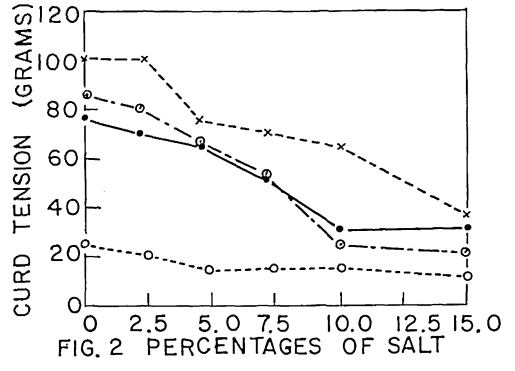


FIG. 2. Effects of salt and starters on the curd tension of pasteurized milk.

Legend:

- ●———● Pasteurized milk + salt and rennet.
- × - - - - × Pasteurized milk + 1% *S. faecalis* + 1 hr. ripening + salt + rennet.
- ⊙—·—·—⊙ Pasteurized milk + 1% *S. lactis* + 1 hr. ripening + salt + rennet.
- ○ · · · · · · ○ Homogenized milk + salt + rennet.

starter of the commercial *S. lactis* resulted in a slight increase in the curd tension of the unsalted milk and the milk containing 2.5% salt. No such effect, however, was observed for the higher concentrations of salt. The increase in curd tension of the milk containing starters (particularly the *S. faecalis* starter) may be due to the acid production by the organisms. The relationship between acid production and curd tension has been pointed out by Heinemann *(4)*. The fact that the curd tension of the samples containing the *S. lactis* starter does not increase for the higher salt concentrations, is probably due to the inhibition of the starter *(1)*.

The effect of the homogenizing process in decreasing the curd tension of milk has been shown by Doan and Welch *(2)* and by Theophilus, Hansen, and Spencer *(8)*. Sanders *et al. (6)* have shown the combined effect of homogenization and pasteurization in reducing the rennet curd tension of milk. Figure 2 shows that the combined effect of homogenization and pasteurization is more pronounced than the combined effect of pasteurization and salt. The addition of salt to the homogenized milk had a slight effect in reducing the curd tension of milk. At this lower level of curd tension, however, the sensitivity of the instrument may be reduced; therefore, the effect of salt may not be detectable.

In connection with the study of the manufacture of Domiati cheese *(7)*, an H_2O_2-catalase treatment was used for the purpose of reducing bacterial count. Therefore, it was considered desirable to determine if this treatment has any effect on the curd tension of milk. For this purpose, a sample of pasteurized milk and a sample of H_2O_2-catalase treated pasteurized milk were compared.

From the results shown (Table 1) it may be seen that the H_2O_2-catalase treatment had no effect on the curd tension of milk.

TABLE 1

Effect of H_2O_2-catalase treatment on the curd tension of milk

Treatment	Time of coagulation 1 hr.	2 hr.	3 hr.
Pasteurized milk + 7.5% salt + rennet	25	50	60
Pasteurized milk + H_2O_2-catalase + 7.5% salt + rennet	25	50	65

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PRELIMINARY RESULTS ON THE PRESERVATION OF BOVINE SEMEN IN A MILK-EGG YOLK-GLYCEROL EXTENDER[1]

The findings of McLean *(3)*, that the addition of glycerol to either egg yolk citrate or boiled homogenized whole milk diluters would prolong the livability of bovine semen, has prompted the rather widespread use of glycerolated extenders for liquid semen. Flipse and Almquist *(2)* found that glycerol at the 5.0% level gave significantly higher spermatozoan motilities in stored semen than did 2.5% glycerol, when each was added to an egg yolk–glycine diluter. More recently, Almquist *(1)* has reported on the use of a milk-glycerol ex-

[1]Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 1792. Approved by the Director.

tender in which the fertility of bovine spermatozoa was satisfactorily maintained for at least four days following collection. VanDemark is reported *(4)* to have found slightly higher conception rates with spermatozoa suspended in a combination of unheated, pasteurized skimmilk, 7:3 yolk-citrate, as contrasted to spermatozoa suspended in a yolk-citrate extender.

Work in progress at the University of Missouri indicates that the use of a combination of heated skimmilk, egg yolk, and glycerol will maintain spermatozoan motility longer than heated skimmilk and glycerol or egg yolk citrate and glycerol. The average of the results for six split-sample storage trials for semen from four different bulls is shown (Figure 1). Postdilution spermatozoan motilities for all samples

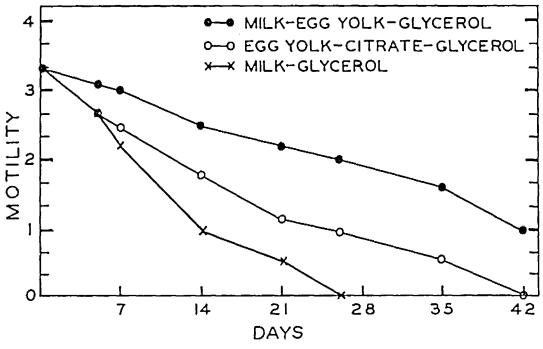


FIG. 1. Postdilution motility of bovine semen stored at from 0 to −3° C. in various glycerolated extenders.

averaged 3 + (0 — 5 motility scale) on the day of collection. Time required for motility to decline to a rating of two (minimum motility rating for semen used in University of Missouri herds) was 8, 12, and 25 days, respectively, for semen stored in heated skimmilk + glycerol, egg yolk citrate + glycerol, and heated skimmilk with egg yolk and glycerol. Progressively motile spermatozoa have been observed in some of the latter samples after 74 days of storage. The diluent was prepared by adding egg yolk (10% by volume) to skimmilk that had been heated (to 95° C.) and cooled. Immediately after collection, semen was diluted to one-half the desired final volume with the milk–egg yolk mixture. The semen-milk-egg yolk mixture was then cooled gradually to 5° C. before final dilution with milk–egg yolk diluent containing 16% glycerol. The glycerolated extender was added in four aliquots at 30-min. intervals. The diluted semen containing 8% glycerol was then placed in 1 cc. plastic vials (vials should be completely filled to minimize exposure to air). The vials were sealed and put in a water bath (5° C.) which, in turn, was placed in a refrigerator for storage at from 0 to −3° C. Freezing point of the extended semen was approximately −5° C., thus making possible liquid semen storage in ice bath.

Preliminary results indicate that the optimum extender composition is approximately 82% skimmilk, 10% egg yolk, and 8% glycerol.

Further studies are in progress on the fertility of semen stored in the milk-egg yolk-glycerol extender and on the factors responsible for the apparent synergistic action of skimmilk and egg yolk constituents in the presence of glycerol.

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FREEZE-DRYING BOVINE SPERMATOZOA

Lyophilization is being used for preserving blood plasma, antibiotics, milk, bacteria, tissue culture media, viruses, and various metazoan cells. Neumann *(4)* found that live tumor cells would withstand freeze-drying. Passey and Dmochowski *(5)* had reported earlier that living tumor cells could be observed only after partial drying. The literature on freeze-drying of spermatozoa of all species is limited. Polge *et al. (6),* using a high-vacuum distillation process on fowl semen, recovered motile spermatozoa. Sherman *(7)* observed negative results with human spermatozoa, when using freeze-drying procedures commonly employed in preparation of materials for cytological studies.

A freeze-drying study was conducted using five bulls (three Holsteins, one Guernsey, and one Jersey). Eight samples were collected and extended 1:4 at 95° F. into whole milk containing 1,000 μg. of streptomycin/ml of extended semen. The extended semen was then

placed in a 400-ml. beaker filled one-third with water at 85° F. and allowed to cool for 20 min. (to approximately 65° F.) at 40° F. Then the extended semen was removed from the water bath and allowed to cool at an air temperature of 40° F. for 1 hr. Commencing at 80 min. postcollection, whole milk extender containing 20% glycerol was added in one-third quantities every 20 min. The semen then had a final extension of 1:8 and contained 10% glycerol per ml. of extended semen. Six of the glycerolated-extended semen samples were allowed to equilibrate for 18 hr. and the remaining two for 10 hr. at 40° F.

After the equilibration period, ⅛-ml. aliquots of glycerolated semen were placed into glass vials suitable for proper shell-freezing (5 ml. tear-drying bulbs). The samples were placed into an inner ethanol freezing bath at 40° F., with the outer isopropanol bath at 30° F. The vials of semen were frozen at the rate of 2.5° F/min to 5° F. Large amounts of dry ice were then added to the inner container, to insure a rapid drop in temperature. When the temperature of the semen reached −60° F., the vials were attached to a Virtis Macro Freeze-Drying Unit. The drying process required approximately 45 min. The vials were removed from the lyophilizer and stored at −110° F. for approximately 10 min. The lyophilized extended semen was restored to original volume by rapid addition of a 3% fructose solution at 100° F. Reconstitution was immediate.

Approximately 5-10% of the spermatozoa in the first five samples of semen from five different bulls showed progressive motility after reconstitution. Though only a small percentage of the spermatozoa recovered their motility, those which did showed very rapid and normal movement. This result suggests that a major portion of the loss in motility was due to physical hazards created during freezing and subsequent desiccation. Spermatozoa in three later samples did not survive, and in each case there was escape of vacuum from the Virtis Lifilizer as the frozen semen-glycerol-whole milk samples were being attached. Also, two of these three samples had been equilibrated for only 10 hr.

Mixner and Saroff *(3)* have found that levels of glycerol above 4% increase the permeability of the living and motile sperm cells, allowing some of them to be counted as dead cells in the differential staining procedure. Therefore, differential staining procedures were not attempted.

The amount of water removed during the freeze-drying process in this study was not calculated. However, Polge *et al. (6)* have estimated 90% of the original water was removed when a distillation process of high vacuum against liquid air was used to desiccate fowl semen, diluted with 30% glycerol in Ringer's solution. From limited data, Polge *et al.* observed up to 30% motile spermatozoa following reconstitution. Bialy and Smith *(1),* using a variety of techniques in freeze-drying bovine semen, observed negative results when dehydration level exceeded 71%. Leidl *(2)* observed living bull spermatozoa following freeze-drying and reconstitution, when the final water content did not go lower than 5% and the glycerol content did not exceed 50%. He observed no motile sperm unless reconstitution was made immediately after completion of the freeze-drying process.

From these limited data, and those of Leidl, it appears certain that bovine spermatozoa can survive freezing and subsequent lyophilization, as evidenced from recovery of limited numbers of progressively motile sperm.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Dr. L. L. Campbell, Jr. and Dr. R. T. O'Brien of the Laboratory of Food Microbiology for their suggestions and for the use of their laboratory equipment.

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OUR INDUSTRY TODAY

MANUFACTURE AND TECHNOLOGY OF SWISS CHEESE [1]

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Swiss cheese, which originated in Switzerland, is often referred to as the King of Cheeses. In addition to Switzerland, the countries which are prominent in the manufacture of this cheese include the United States, Finland, and Denmark. In its home country, the cheese is known as Emmentaler cheese; in other countries, as Swiss cheese. Genuine Swiss cheese has a flavor and aroma which cheese-makers in other countries have had difficulty in duplicating. This superiority has generally been credited by cheese-makers to the aroma of certain alpine grasses, which is imparted to the milk and then becomes a part of the cheese flavor. But exact duplication of all of the many variables involved in manufacturing and ripening probably would result in Swiss cheese comparable in flavor to that of Emmentaler.

Characteristics of Swiss Cheese

Swiss cheese, as defined in the Federal Register, is the food prepared from cow's milk by the so-called Swiss Cheese Procedure. It has holes or eyes developed throughout the cheese, contains not more than 41% moisture and not less than 43% milk fat in the dry matter, and is not less than 60 days old.

Four grades are recognized in the United States Standards for Swiss cheese. Grade A Swiss is characterized by being free from off-flavors. In addition, it should possess some degree of characteristic Swiss cheese flavor, depending on its age. The body should be uniform, firm, and smooth. The eyes should be round or oval and uniformly distributed, with the majority not being over ½ in. in diameter. A plug of cheese taken with a No. 8 trier should show from one to eight eyes. The color should be uniform and the rind should be dry and free from defects.

Swiss cheese of Grades B, C, and D quality deviates from Grade A cheese in one or more of these standards. The greater the deviation, the lower the grade of the cheese.

Down-grading of Swiss cheese usually results from the use of inferior milk, or because of improper acid development during making. Such conditions may result in cheese with a spongy or gassy body and faulty eye development. Over-set, rough-eyes, dead-eyes, and blind are some of the terms used to describe abnormal eye formation. Off-flavors encountered in defective Swiss cheese may include fermentation flavors such as fruity, sour, and yeasty. Flavors such as weed and onion can be attributed to feeding conditions.

Manufacturing Procedure

Swiss cheese is traditionally manufactured in copper kettles. This is in contrast to other types of cheese, which are manufactured in vats of more inert materials, such as tin-plated metal or stainless steel. A kettle may contain from 2,500 to 3,500 lb. of milk, sufficient to make one Swiss cheese ranging in weight from 160 to 250 lb.

Milk with a methylene-blue reduction time from 4 to 5 hr. is usually satisfactory for the manufacture of Swiss cheese. The milk is partly skimmed, because whole milk often results in cheese of inferior body and texture characteristics, causing the defect termed as glass. Raw milk is used by most factories, although milk heated to less than pasteurization temperature is used advantageously in many places. Attempts to make Swiss cheese from completely pasteurized milk have not been entirely successful.

The following is a schematic outline of the steps generally involved in the manufacture of Swiss cheese. Slight adjustments in the procedure may be necessitated by the quality of the milk, the cultures used, and the season of the year.

Addition of water to the milk before setting is a common procedure in Europe, but is not practiced in this country. The procedure results in a cheese with a more elastic body, because of the dilution of the lactose and a subsequent reduction in lactic acid formation. However, it requires the best of milk and a pure water supply.

The recommended amounts of starters pertain to milk-starters. Almost twice these amounts are required when whey-starters are used. The cultures are added before the rennet, and the milk is frequently allowed to ripen for about 30 min., because most cheese-makers want slight acid development before adding rennet.

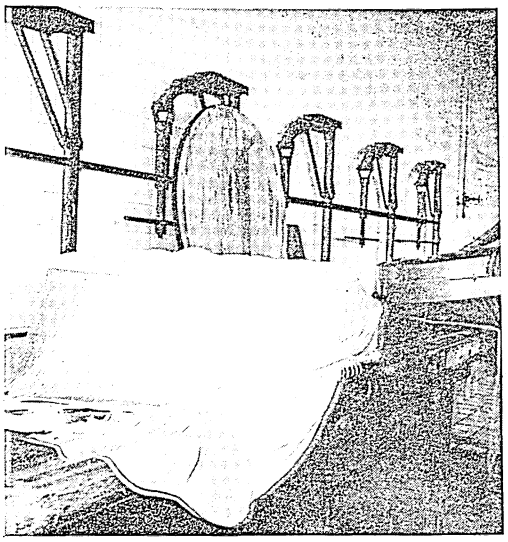
The rather high setting temperature makes it necessary for the cheese-maker to watch the curd closely, to determine the proper time of harping (cutting). If harping is delayed, it

[1] Article No. 13-57. Department of Dairy Technology, The Ohio State University.

Schematic Outline of Procedure for Manufacture of Swiss Cheese

Cultures
Rod—0.05–0.1%
Coccus—0.05–0.1%
Propionibacterium—1 ml/100 lb. milk

Water	0–5%
Rennet	2–3 oz/1,000 lb. milk
Setting temperature	90°–92° F.
Setting time	25–30 min.
Cutting (harping)	10–15 min.
Foreworking	20–30 min.
Heating temperature	122°–124° F.
Cooking	30–40 min.
Stirring out	30–40 min.
Turned on press, first day	4 times
Dry salt	24 hr.
Brinesalt	48–72 hr., 54–56° F., 22% brine
Cold curing room, 56° F.	12–14 days
Warm curing room, 68–74° F.	5–7 wk.
Cold curing, 54–56° F.	Until sold



—Courtesy Town Hall Cheese Co., Wis.

FIG. 1. Hooping of wheel style of Swiss cheese, showing texture of freshly dipped curd.

may be impossible to get a fine curd. This may result in high-moisture cheese. Heating should be conducted with care. Rapid heating may result in formation of a film on the curd particles which would retard, or prevent, proper expulsion of whey.

Temperatures lower than 122-124° F. prolong the cooking time and bring about too rapid acid development in the vat. Temperatures above 122-124° F. adversely affect the acid-producing bacteria. It has been demonstrated experimentally that the higher the temperature of cooking over 124° F., the poorer the quality of the cheese. The major criticism of such cheese was excessive eye-formation.

The firmness of the curd before hooping determines the moisture content and the amount of acid which will be formed in the cheese, thereby establishing the body and texture characteristics of the cheese (Figure 1).

The dipping process consists of removing all of the curd from the vat in one mass. Care must be taken to insure a compact mass of curd in the hoop. If the interior is broken, a whey-pocket may result. Such a pocket is almost impossible to press out, and eventually abnormal fermentations will occur.

Pressure should be applied slowly to the cheese on the press, to allow whey to escape. When turning the cheese, care should be taken not to tear the surface of the cheese if the cloths stick.

Salting and curing to some degree determine the appearance and quality of the cheese. Some cheese-makers favor a slight dry-salting for 24 hr. before placing the cheese in the brine; whereas, many use only brine salting. The use of dry salting results in a better rind formation. Salt is inhibitory to eye-formers, which makes it possible to control these bacteria by varying the final salt concentration in the cheese.

After the cheese has been salted, it is allowed to dry for a day and is then moved to a cold room, where it is placed on shelves on circular boards. The temperature in the cold room ranges from 50 to 54° F. Salt is sprinkled on the surface of the cheese two or three times a week and the surface is then scrubbed and wiped off, to prevent surface growth (Fig. 4).

After about 2 wk. at this temperature, or when the texture of the cheese has become sufficiently elastic to support eye-formation, the cheese is transferred to storage at from 68 to 74° F. The treatment of the surface is continued, because only little surface growth is desirable until the cheese is about 6 wk. old. The propionibacteria grow in the warm room and eye formation results. After about 5 wk., eye formation usually is complete. This can be tested with a trier and by observing the appearance of the cheese. The cheese is then moved to a cold room at about 52° F., where curing is completed.

The curing room should be kept at from 85 to 90% relative humidity, to prevent excessive moisture loss. The cheese should be turned twice a week to prevent rind rot.

Modifications of the Swiss Cheese Manufacturing Process

A relatively recent development, which has revolutionized the entire Swiss cheese industry in the United States, is the introduction of rindless block Swiss cheese. Two principal methods

for the manufacture of this type of cheese are employed.

One method utilizes stainless steel vats of up to 20,000-lb. capacity for making the curd, rather than the conventional copper kettles. When the curd is sufficiently firm, the whey and curd are pumped to a special device which serves as strainer and hoop, where the curd is collected. The curd is then pressed and the large block of cheese is cut into smaller blocks of more convenient size. After the cheese has been salted, the surface of the cheese is dried. The protection of the surface is afforded by placing the cheese in a large plastic bag. The air is evacuated from the bag, the bag is heat-sealed, and the entire unit dipped in cheese-wax as an added precaution against air leaks.

The second method of manufacture of rindless block Swiss cheese calls for the cheese being made in the conventional copper kettle. After the curd is firmed sufficiently, the curd mass is transferred to a square hoop and pressed (Figure 2). The next morning, the

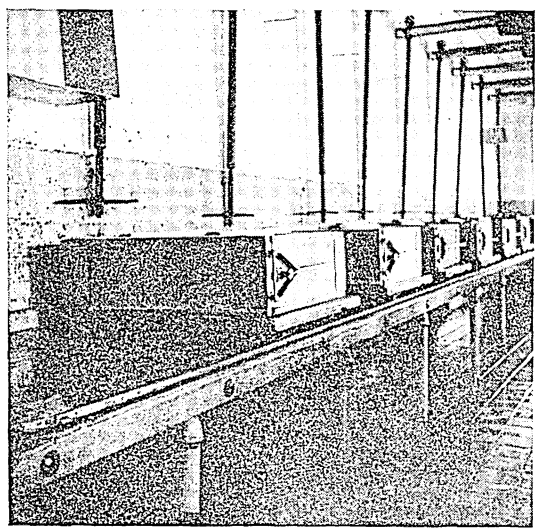


FIG. 2. Pressing of block-style Swiss cheese.

square cheese is cut into two pieces and brine salted. After salting, the surface of the cheese is dried and a thin layer of petrolatum applied. The final protection of the cheese surface is brought about by wrapping the cheese in a piece of plastic material, which is sealed by tape only.

After wrapping, rindless block Swiss cheese is placed in wooden or carboard boxes which are large enough to allow for upward expansion of the cheese. Curing takes place as for regular wheel Swiss, with occasional turning being the only treatment needed.

Rindless block Swiss cheese has definite economic advantages over the conventional wheel cheese, in regard to handling and merchandising. Whereas the appearance of the cut cheese is little different from that of the wheel Swiss, the flavor which develops in the wrapped cheese often is lacking, compared to that of regular Swiss.

Technology and Science of Swiss Cheese Manufacture

Cultures. Three different bacteria are used as cultures in Swiss cheese making: *Streptococcus thermophilus* (the coccus), *Lactobacillus bulgaricus* (the rod), and *Propionibacterium shermanii* (the eye former).

S. thermophilus and *L. bulgaricus* are generally selected on their acid-producing capacity. Little or no attention is given to the contribution they may make to flavor development. *S. thermophilus* is responsible for the acid produced during the first 7 to 8 hr., and *L. bulgaricus* is responsible for the acid developed from 8 to 21 hr. After about 21 hr., acid development in the cheese is completed.

The role of *S. thermophilus* during the ripening process is not entirely understood, whereas the role of *L. bulgaricus* is assumed to be the break-down of casein.

Proper care of cultures, to maintain actively growing strains and to avoid contamination, is an important part of Swiss cheese manufacture. Mother cultures of *S. thermophilus* and *L. bulgaricus* should be propagated every day. Some cheese-makers prefer to carry the cultures in milk, others prefer whey. Whey has some advantage, because it contains certain growth-promoting factors developed during cheese manufacture. However, when whey is used, great care must be exercised to assure destruction of bacteriophage by sterilization. The medium should be autoclaved for 15 min. at 15 lb. pressure, or at least heated by steam or water to approximately 200° F. for 1 hr. For best results, the medium should be made fresh every day. After it has been heated, the milk or whey is cooled to 98-100° F., inoculated with 0.1% culture, and placed in an incubator at 98-100° F.

Milk may be used as the medium for bulk starter and should then be heated to 190° F. for 1 hr. and cooled before inoculation. Frequently, however, whey is used for this purpose. The whey is pasteurized at 150-160° F. for 30 min., cooled and inoculated with the acid-formers, either singly or in combination. Bulk starters should also be incubated at 98-100° F.

An incubation time of 16-18 hr. is usually satisfactory. Milk cultures of Lactobacilli should then have an acidity of 1.1-1.25%, and milk cultures of Streptococci should have an acidity of 0.75-0.80%. Whey cultures will usually have about 0.4% less acid than milk cultures.

After incubation, mother cultures should be cooled to 55-50° F. until transferred. Bulk starters should be used within a few hours. Prolonged incubation results in a decrease

in the rate of acid formation which could affect the quality of the cheese.

P. shermanii may be carried in a simple medium of 1% lactate, plus 1% yeast extract, in distilled water adjusted to pH 6.6. Media containing whey or whey powder may also be used. In general, media containing some lactate are better than media containing only lactose as the major source of carbon, because these bacteria do not readily ferment lactose. The medium should be autoclaved, as described previously for milk. After inoculation, the cultures are incubated at about 86° F. for from two to three days in lactate media, or from five to seven days in lactose media. These cultures remain viable for weeks and do not require daily transferring.

Changes During Ripening

Experience has demonstrated that the probability of getting the best flavor developed in Swiss cheese is when the pH of the cheese reaches 5.16 to 5.25 within 14-16 hr. after addition of rennet. Deviations from this usually result in cheese with poor eye formation and flavor. As the cheese ripens, pH increases and reaches a value of from 5.70 to 5.80 after about 6 mo. of curing. The increase is rather uniform throughout the ripening period.

The fermentation of part of the lactate to propionate and acetate, and the degradation of proteins which takes place during curing, may be contributing factors to the increase in pH.

Propionic acid bacteria are generally credited with the fermentation of lactate, with flavor and eyes developing concurrently. The over-all fermentation involves:

$$3\ CH_3CHOHOO^- \longrightarrow 2\ CH_3CH_2COO^- + CH_3COO^- + CO_2 + H_2O$$

Lactate → Propionate + Acetate + Carbon Dioxide + Water

The exact mechanism of the reaction is still debated. The carbon dioxide formed during the fermentation accumulates in weak spots in the cheese and forms the eyes.

That propionic acid bacteria are not responsible for all of the carbon dioxide present in Swiss cheese was recognized as early as 1912 by Clark *(2)*. He found that the concentration of carbon dioxide in Emmental cheese was much higher than could be explained by the propionic acid fermentation alone. He distinguished between a saturating and an inflating gas production. The former was presumed to arise from carbohydrate fermentation by lactic acid bacteria and to be equally as important to eye formation as the latter, arising from the propionic acid fermentation.

Recent research in this department has demonstrated that a bacterial fermentation system related to the citric acid cycle is functioning in Swiss cheese. One of the products of this cycle would be carbon dioxide, thus confirming Clark's findings.

Theoretically, the propionic acid fermentation should yield propionic and acetic acid in a ratio of 2:1. Such a ratio rarely, if ever, exists in Swiss cheese. The ratios encountered in this laboratory have ranged from 1.6:1 to 0.5:1, with ratios of 1.5:1 to 1.2:1 being more common.

Succinic acid has been demonstrated to be one of the products of the propionic acid fermentation, but the concentrations demonstrated in Swiss cheese have not been sufficient to account for the low ratios of propionic to acetic acid.

A more likely explanation for the low ratios could be that acetic acid originates from other reactions in addition to the propionic acid fermentation. Decarboxylation of pyruvic acid, which is present in Swiss cheese in higher concentrations than in any other variety, would yield acetic acid. The amino acid glycine would yield acetic acid if deaminated.

In addition to propionic and acetic acid, Swiss cheese also contains butyric and higher volatile fatty acids, including valeric acid. Milk fat, carbohydrate, and amino acids all have been suggested as possible sources of these fatty acids. The role of these acids is not too well understood, although it has been established that excessive concentrations of butyric acid are associated with inferior cheese.

With the advent of comparatively simple but highly accurate methods for the separation and quantitative determination of free fatty and amino acids, it was discovered that propionic and acetic acid are not entirely responsible for the characteristic flavor in Swiss cheese. Analyses demonstrated that cheeses with high and low flavor intensities could have identical levels of these acids. Correlation of propionic acid contents with free amino acid contents showed that high concentrations of the amino acid proline and/or the conditions leading to high concentrations of proline were as essential to characteristic flavor as high levels of propionic acid. Aged Swiss cheeses lacking in flavor were low either in proline or in propionic acid, indicating the importance of a proper balance of both of these compounds for characteristic flavor.

Analysis of wheel and rindless block Swiss cheese has contributed further evidence to the role of a high concentration of proline to the development of characteristic Swiss cheese flavor. Wheel Swiss contained several times as much proline as did rindless cheese. The two types of cheese contained approximately the same concentrations of propionic and acetic acids. The wheel Swiss cheese was considered typical in flavor; whereas, the rindless was criticized for being lacking or atypical and putrid. The rindless block Swiss cheese included

in this study was made in copper kettles and the plastic wrapper sealed with tape only.

The occurrence of free amino acids is caused partly by break-down of casein by *L. bulgaricus* and perhaps also by *S. thermophilus*. Some free amino acids may originate through transamination, involving glutamic acid and keto acids from carbohydrate or citric acid fermentation. The contribution of propionibacteria to the amino acid pattern of Swiss cheese has not been established. That this bacterium in some manner is responsible for the high levels of proline, either singly or in symbiosis with the two lactic acid cultures, cannot be excluded. Other cheese varieties, where *S. thermophilus* and *L. bulgaricus* are used as acid-producers, are not characterized by high levels of proline.

Besides the previously mentioned compounds, a number of trace compounds are associated with the flavor of Swiss cheese. This contribution is either indirect, the compounds being intermediary in fermentation cycles, or direct, the compounds being end-products of fermentation. The exact status of many of these compounds is difficult to establish. Perhaps it is safe to assume that all compounds present contribute to the flavor of cheese, whether they be considered intermediary or end-products.

The more prominent of the trace compounds identified in Swiss cheese include pyruvic acid and members of the previously mentioned citric acid cycle. The member compounds include oxalsuccinic, oxalacetic, alpha-ketoglutaric, glyoxylic, and alpha-acetolactic acids.

The presence of these compounds in fresh cheese can be explained on the basis of the fermentation of citrate. The increases in concentration of these compounds during curing are not so readily explained, since citrate presumably is exhausted during the first few weeks of ripening. Keto acids which are derived from amino acids and fatty acids in other varieties of cheese are generally absent in Swiss cheese. However, iso-keto-caproic acid which could originate from iso-leucine has been isolated from some Swiss cheeses.

In addition to the acidic carbonyl compounds, a number of neutral carbonyl compounds are present in Swiss cheese. Some have been identified, such as diacetyl, acetylmethylcarbinol, and acetone. A number of longer chain compounds present have not yet been identified.

The major fermentations characterizing Swiss cheese are shown (Figure 3).

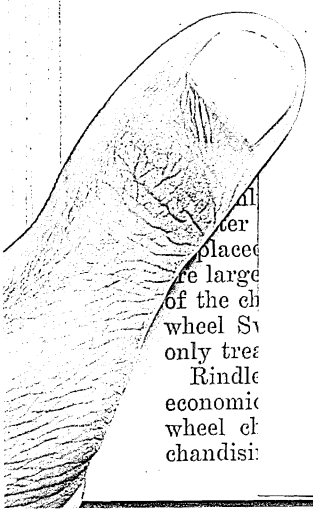
Role of Copper in Swiss Cheese

Swiss cheese is the only variety of cheese traditionally manufactured in copper vats, exclusive of a few very closely related varieties. All other varieties are made in vats having a relatively inert surface, such as tin or stainless steel, as previously mentioned. With the advent of stainless steel for the manufacture of dairy equipment, attempts were made to make Swiss in stainless steel vats. So far, these attempts have met with mixed success. The flavor and body characteristics of Swiss cheese made in stainless steel vats generally are considered inferior to those of Swiss cheese made in copper kettles.

Swiss cheese made in copper kettles contains about 17 p.p.m. of copper. The presence of as little as 3 to 4 p.p.m. of copper is usually detrimental to other varieties of cheese. The body and texture of Swiss cheese are quite different from those of other hard varieties.



FIG. 2.



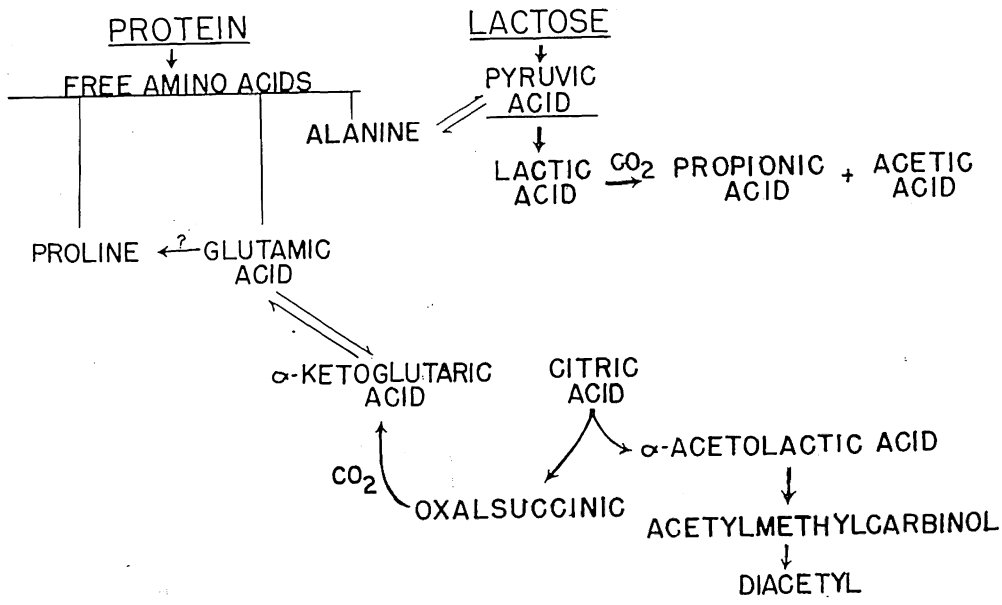


FIG. 3. Major fermentation pathways in the ripening of Swiss cheese.

To a large extent this can be attributed to differences in manufacturing procedure and cultures, but copper, perhaps by combining with protein, probably plays a role in bringing about the desired characteristics. At least, Swiss cheese made in stainless steel vats often has body and texture characteristics different from Swiss made in copper kettles, and does not support proper eye formation. "Slits" rather than eyes often occur in cheese made in stainless steel kettles.

The effect of copper on flavor development may be indirect. High-grade Swiss cheese, more than any other cured variety, is void of proteolytic flavor. From studies on milk, it is known that copper reacts with sulfhydryl groups, and greatly reduces the number of these groups exposed by subsequent heat treatment. The role of copper in Swiss cheese may be similar—to react with sulfhydryl groups in such a manner as to prevent extensive exposure of active sulfhydryl groups and release of hydrogen sulfide during ripening.

Recent work in Switzerland *(1)* indicated that proteolysis during ripening was more extensive in Swiss cheese made in stainless steel vats than in cheese made in copper kettles; however, the amount of soluble nitrogen formed was largest in the Swiss cheese made in copper kettles. Also, the propionic acid fermentation appeared to be affected adversely in cheese made in stainless steel vats, particularly in the later stages of ripening. These results could indicate that in Swiss cheese made in stainless steel vats, the exposure of more sulfhydryl groups, with the possible release of more hydrogen sulfide, interferes with the propionic acid fermentation.

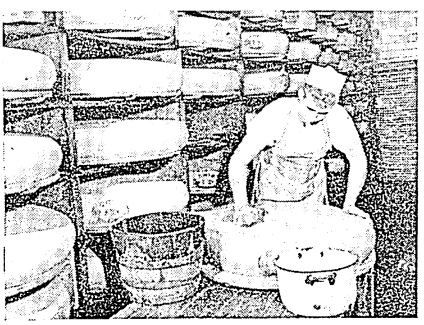


FIG. 4. Surface care of wheel-type Swiss cheese in the warm curing room.

Summary

The successful outcome of Swiss cheese manufacture is dependent on a number of factors. Acid-forming and eye-forming bacteria have to be selected and properly taken care of by frequent propagations. Every step involved in the manufacturing procedure must be applied in a manner which will insure a proper rate and amount of acid development.

During curing, degradation of the constituents of the cheese occurs. Fatty acids and amino acids are liberated. Certain compounds important in intermediate bacterial metabolism also are formed.

Characteristic flavor and body and texture development of Swiss cheese appear to be dependent on a proper balance of products of metabolism. Poor milk, improper rate of acid development, and adverse curing-room conditions are some of the causes of low-quality Swiss cheese. Certain theories are advanced regarding flavor development and the role of copper in Swiss cheese.

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OUR ASSOCIATION

SCORE-CARD AND GUIDE FOR COTTAGE CHEESE[1]

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The scoring guide enumerates the common defects and the suggested score. This guide should assist in standardizing the grading of cottage cheese for contests or for routine plant quality control. The suggested ranges for the defects are based largely on 20-yr. averages of judges' scores of other dairy products at the International Students' Contests. The terms which have been used are defined, in order to assist in describing the defects. The product is considered unsalable if it is scored below the normal range.

[1]Published according to the policy stated, *J. Dairy Sci.*, 40: 1369. 1957.

Ideal cottage cheese. The flavor of cottage cheese should be similar to fresh, clean skimmilk or cream (if creamed). Cottage cheese should have a slightly acid, salty taste, with the delicate flavor and aroma of a good lactic starter (diacetyl). The body of cottage cheese should be uniform, smooth, and meaty (not too firm or too soft and pasty). The curd particles

Scoring guide for cottage cheese[a]

Flavor defects	Slight	Distinct	Strong	Excellent 45–40 No criticisms	Good 39.5–38.5	Fair 38–36.5	Poor 36–32	Bad 31.5–0
Flat	39.5	39	38.5	Normal	Sl.[b] D.S.			
Feed	39	38.5	37.5	Range	Sl.D.	S.		
High salt	38.5	38	37	42–32	Sl.	D.S.		
Acid	38.5	38	36		Sl.	D.	S.	
Storage	38	36	34			Sl.	D.S.	
Sour	37	34	32			Sl.	D.S.	
Medicinal	37	34	31			Sl.	D.	S.
Foreign	37	34	31			Sl.	D.	S.
Stale	36.5	32	29			Sl.	D.	S.
Bitter	36	32	28				Sl.D.	S.
Unclean	36	31	27				Sl.	D.S.
Malty	35.5	30	25				Sl.	D.S.
Metallic	35	29	24				Sl.	D.S.
Fruity or fermented	35	28	23				Sl.	D.S.
Musty	34	27	22				Sl.	D.S.
Oxidized	33.5	25	18				Sl.	D.S.
Yeasty	33	24	15				Sl.	D.S.
Rancid	32	23	14				Sl.	D.S.

Body and texture defects	Slight	Distinct	Strong	No criticisms Excellent 35–34.5	Good 34–32.5	Fair 32–30	Poor 29.5–0
Firm or hard	34	33	32	Normal	Sl.D.	S.	
Weak	33.5	32.5	31.5	range	Sl.D.	S.	
Mealy	33	32	31	34.5–31.0	Sl.	D.S.	
Rubbery	32.5	31.5	30		Sl.	D.S.	
Pasty	32	31	28			Sl.D.	S.
Gelatinous	31.5	29	26			Sl.	D.S.

Color and appearance defects	Slight	Distinct	Strong	Excellent 15–14.5 No criticisms	Good 14–13	Fair 12.5–11	Poor 10.5–0
Lacks uniformity	14.5	13.5	12.5	Sl.	D.S.		
Overcreamed	14	13	12	Normal	Sl.D.	S.	
Lacks cream	13.5	12.5	11.5	range	Sl.	D.S.	
Shattered curd	13	12	11	14.5–11.0	Sl.	D.S.	
Matted	13	12	11		Sl.	D.S.	
Unnatural color	12.5	11.5	10			Sl.D.	S.
Free whey	12	11	9			Sl.D.	S.
Translucence	11.5	10	8			Sl.	D.S.
Surface discoloration	11	9	7			Sl.	D.S.
Slimy	10	8	5				Sl.D.S.

[a]Defects listed in order of most objectionable.
[b]Sl.—Slight (barely detectable), D.—Distinct, S.—Strong.

should be discrete, uniform in size (regardless of style), and of a natural, creamy-white color. Creamed cottage cheese should have a uniform layer of cream around the curd particles, with a minimum of free cream. Any excess cream should be of a thick consistency (not whey-like or watery).

TERMS USED TO SCORE COTTAGE CHEESE

Flavor—45—Normal Range, 42–32

Bitter—An astringent, puckery taste.
Feed or weed—The aromatic flavor characteristic of certain feeds and weeds.
Flat—Lack of characteristic flavor.
Foreign—Any objectionable flavor not characteristic of the product and not specifically listed.
Fruity or fermented—The aromatic flavor of orange, pineapple, strawberry, etc., usually due to bacterial action.
High acid—An excess acid over and above that considered desirable, but which is clean and sharp.
High salt—Excessive salt over that desired by consumer.
Malty—A malt-like flavor due to bacterial action.
Medicinal-Chemical—The smell and taste of disinfectant, chlorine, iodine, or phenolic compounds.
Metallic—A metal taste. Astringent. (A similar taste is noted when a penny is placed in the mouth.)
Musty—The odor of a damp, poorly ventilated cellar, or refrigerator.
Oxidized—A cardboard flavor.
Rancid—The pungent, rasping aroma and taste of butyric acid.
Sour—Excessive acid usually associated with other bacterial defects, such as fruity, fermented, etc., and is not clean.
Stale—A lack of freshness.
Storage—Odors absorbed from storage rooms or refrigerators.
Unclean—Reminiscent of the odor of dirty equipment, possibly due to inferior milk.
Yeasty—The aromatic flavor, characteristic of any fermentation by yeasts.

Body and Texture—35—Normal Range, 34.5–31.0

Gelatinous—A sticky, jelly-like character.
Mealy—A character of curd which resembles corn meal in the mouth.
Pasty—A soft, weak curd which resembles a flour-water paste.
Rubbery—An elastic, resilient curd.
Too firm—A curd which is hard and does not break easily in the mouth.
Weak—Curd which is soft and lacks body.

Color and Appearance

15—Normal Range, 14.5–11.0

Free whey—The presence of a thin, watery fluid.
Lacks cream—A dry curd lacking sufficient cream.
Lack uniformity—Lack of uniform-sized particles.
Matted—The agglomeration of two or more particles of curd.
Overcreamed—The presence of too much free cream.
Shattered curd—Curd which has been broken, owing to improper handling or packaging, and which may exhibit curd dust.
Slimy—A slippery film on the curd surface, due to chemical or microbial agents.
Surface discoloration—A noncharacteristic color on the surface, due to the growth of microorganisms.
Translucent—A lack of opaqueness, caused by chemical or microbial agents.
Unnatural color—Curd which does not present a creamy-white color.

Package—5—Normal Range, 5–4

Broken—A broken, torn, or damaged carton.
Container not full—A carton not containing the declared amount of cheese, or not full within ½-in. from the cover seat.
Insanitary closure—In which the closure is inset in the carton and not over the lip of the carton.
Leaky—A nonwaterproof carton, or one which leaks whey or cream. A soggy carton.
Soiled—A carton or closure presenting an unattractive exterior appearance, because of the presence of cheese, cream, or foreign matter.

REPORT OF REPRESENTATIVES OF THE AMERICAN DAIRY SCIENCE ASSOCIATION ON THE DAIRYWIDE COORDINATING COMMITTEE FOR NUTRITION RESEARCH[1]

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K. G. Weckel
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The Dairywide Coordinating Committee on Nutrition Research is composed of representatives of 15 associations, representing both dairy products manufacturers and producers' groups.

[1]R. G. Hansen usually served as Chairman, but he was in Europe at the time this report was prepared; therefore, it was submitted by J. D. Ingle and K. G. Weckel.

The Chairmanship of the Committee is rotated annually, alphabetically, among the participating organizations. Since January, 1957, the Committee has served under the chairmanship of H. F. Judkins, Sec.-Treas. of the American Dairy Science Association.

The objectives of the Dairywide Coordinating Committee are to provide assistance toward

better coordination of nutrition research in the dairy industry, especially toward greater use by the industry of established facts in nutrition. Specifically, they are:

1. Assembling and evaluating from available sources, consumer and professional attitudes toward dairy foods
2. Reviewing scientific data to provide factual background for public information about dairy foods
3. Suggesting additional needed nutrition research to encompass all phases of human nutrition as affected by dairy foods
4. Presenting to the dairy industry authentic information about dairy foods, and formulating recommendations for use in public relations, advertising, and educational work
5. Making available to dairy groups an annual summary of dairy industry–sponsored nutrition research projects in progress.

The operating headquarters of the Committee are in the offices of the National Dairy Council, which has furnished space and considerable staff and materials assistance.

The Committee operates most effectively through a number of subcommittees. The Committee meets twice yearly, in January and June, and the subcommittees more frequently. The subcommittees consist of *(a)* a research survey subcommittee; *(b)* an editorial subcommittee, to study the recommendations of the research survey subcommittee, to determine subject matter for articles for industry.

In 1953, a Subcommittee on Attitudes poll submitted a report on consumer attitudes, which has served as a basis of much of the Committee's attention. The principal points in the poll on attitudes were: *(a)* fat metabolism (reducing diets); *(b)* diseases; *(c)* effects of heating on milk; *(d)* calcium assimilation; *(e)* dental caries; *(f)* malnutrition, and *(g)* cholesterol. Three other phases of the poll were classified as: nutrition education, produce promotion, and field of economics.

The Research Survey Subcommittee in 1954 reported the following as subjects on which good information could be obtained, which could be used by the dairy industry for promotion: *(a)* Dairy foods in weight-reduction diets; *(b)* selling dairy foods on their protein value (R. G. Hansen, agreed to prepare three articles for use by industry, and the editorial subcommittee drew plans for effective merchandising of the articles in industry); *(c)* dairy foods for dietary fat; *(d)* dairy foods for malnutrition; *(e)* dairy foods in dental health, for calcium, and in utilization of calcium.

It presented a report of a Bibliography of Scientific Reports of Dairy Industry–Sponsored Nutrition Research.

In 1954, a report was made by the Research Survey Subcommittee on suggestions for a nutrition research program for the dairy industry. This report rather detailed and lengthy covered gaps in the information on milk and human nutrition, butterfat and fat-soluble factors, and whole milk and water-soluble nutrients.

In 1955, the Research Survey Subcommittee issued its revised report on "Recommendations for Nutrition Investigations of Primary Importance to the Dairy Industry." This was submitted to the Editor of the *Journal of Dairy Science,* who recommended certain revisions. Consideration was given to these suggestions by committee action. In the fall of 1955, Dr. Hansen issued a paper "Milk Protein—Invaluable Source of 'Body Building Blocks'," for release through the Committee. The Committee released also "Thirty-three Dairy Industry–Sponsored Nutrition Research Projects."

At the June 28, 1956 meeting, consideration was given to further recommendations for nutrition research, including human requirements of less well-known but well-studied essential nutrients, butterfat in production and processing, comparison of lysine-enriched bread and bread with nonfat milk solids, relative digestibility of butterfat and other fats, galactose metabolism and galactocemia, and infant feeding with reference to amino acid composition.

A second article by Dr. Zoe Anderson, "The Role of Dairy Foods in Weight Reduction and Control," was released through the Committee to the press for industry use.

In June, 1956, a special subcommittee was appointed to make recommendations to guide industry in using nutrition information in promotional programs and for establishing a recommended policy for ethical advertising in promoting dairy products. There appear to have been numerous instances of exaggerated or unsupported claims by the dairy industry for dairy products as nutritive foods. This subcommittee developed a proposed policy for consideration by the general committee at the June, 1957 meeting.

In July, 1956, the Research Subcommittee participated in a survey to help acquire information for bringing up to date, and more completely, the Agriculture Handbook No. 8, "Composition of Foods—Raw, Processed, Prepared," published by the U. S. Department of Agriculture and scheduled for revision.

The Research Subcommittee gave considerable thought to current nutrition and health problems affecting the dairy industry, including: *(1)* Implication of fat as a causative factor in heart disease, *(2)* alleged presence in milk of a virus acting as a causative factor in heart disease and cancer, *(3)* alleged presence of cancer-causing materials in dairy products resulting from carcinogens in plastic-coated milk containers and carcinogens in plastic coatings of containers for butter and cheese, and yellow coal-tar dyes, *(4)* implications of antibiotics in milk, and *(5)* utilization of milk calcium.

Members of the Subcommittee have participated in conferences at Loyola University, and in Chicago on atherosclerosis and fats in the

diet. This subject is currently occupying a considerable portion of the efforts of the Research Subcommittee and of the Editorial Subcommittee on ways and means of providing the dairy industry with information to use in answering the public's questions. A meeting of the Coordinating Committee was scheduled for June of 1957.

The representatives of the American Dairy Science Association are members of the subcommittees of the Committee. The work of the committees, being entirely voluntary, is seemingly slow; the problems are ponderous, but apparently are best met through such means. Continued participation on the Committee by A.D.S.A. is recommended by its present representatives, all of whom have participated in at least six meetings during the year.

The résumé of the period prior to the past year has been presented solely to provide the Executive Board of A.D.S.A. with a background for current activities.

REPORT OF MEETING OF GOVERNING BOARD OF AMERICAN INSTITUTE OF BIOLOGICAL SCIENCE

H. D. Eaton[1]

Connecticut Agricultural Experiment Station, Storrs[2]

The meeting of the Governing Board of the American Institute of Biological Science was held in the United Nations Room of the Student Union at the University of Connecticut, on August 25, 1956, and was called to order by President Glass.

Executive Secretary's report. After a few introductory remarks by President Glass, the Executive Secretary, Dr. H. T. Cox, gave his report. As an example of the possible service to member and affiliate societies, he indicated the role of A.I.B.S. in obtaining funds from the National Institutes of Health for the preparation of a 15-year Cumulative Index of the *Journal of Parasitology.* He further emphasized that A.I.B.S. wants to know the needs of its members and affiliate organizations so that it may do a better job. After Dr. Cox's report, methods of communicating the report to the membership, as well as the general purposes of A.I.B.S., were discussed.

1956 Activities of A.I.B.S. President Glass briefly indicated the many activities A.I.B.S. had undertaken during the past year. For example, he cited the aid of A.I.B.S. in providing travel for 40 U.S. scientists to the International Conference on Genetics held in Japan. A.I.B.S. also selected the persons who attended.

Authorized new activities. 1. *General Conference of Editors.* Based on recommendations of a preliminary conference of a small number of editors of biological journals, a proposal prepared by A.I.B.S. was submitted to the National Science Foundation (N.S.F.) for consideration, in May of 1956. This proposal indicated a conference of 50 editors in all fields of biology, except those medically oriented, with expenses for travel and subsistence to be paid by A.I.B.S. with funds provided by N.S.F. This conference was approved by N.S.F. and was scheduled for early in 1957.[3]

2. *Conference on Aging as a Biological Problem.* This conference was currently being evaluated by N.S.F.

3. *Visiting Lecturers' Programs.* A grant of funds from N.S.F. and the Atomic Energy Commission (A.E.C.) has provided A.I.B.S. with sufficient funds to provide 70 weeks of lecturing. The plan, in brief, is to provide a list of lecturers who are not only respected for their scientific status but who can also present biology in a favorable manner ("sell biology"). These lecturers will receive travel allowances and a modest honorarium, with the host institution providing lodging and meals. This program is intended for small liberal arts colleges and nearby institutions, e.g., high schools, biology clubs, etc. The duration of the lecturer's residence at the institution would not be less than three days and not more than seven. This program is on a year-to-year basis, its renewal dependent upon results. To date, biologists have viewed it with considerable favor.

After presenting these new activities, the selection of editors, conferees, and lecturers for these programs was discussed. This was based on the necessity of limiting the number for each program. It appeared to be the consensus, but was not unanimous, that some announcement of these programs should be made, for example in *Science,* for the purpose of allowing all those interested to apply. However, final selection will rest with the Committee charged with the duty of selection and in no way should limit the committee's selection only to those who applied. After further discussion

[1] Representative of the American Dairy Science Association, an affiliate of the American Institute of Biological Science.

[2] Published according to the policy stated, *J. Dairy Sci.*, 40: 1369, 1957.

[3] This conference for biological editors was held in New Orleans, Louisiana, April 2–4, 1957. Problems considered were those relating to the economy and management of scientific journals, the selection and preparation of material for publication, the cooperation of journal editors with indexing and abstracting services, and the need for new publication media. *E. O. Herreid.*

as to whether or not this procedure should be put in the form of a motion, it was indicated that these were thoughts of the majority of the Governing Board and should be considered by the Executime Director in carrying out such programs.

Proposed activities. 1. *Quarterly Review of Biology.* Since the present editors of this Journal felt that they could no longer operate under the conditions imposed by Williams and Wilkins, the publishers, and the Waverly Press, printers (minimum need was one full-time editorial assistant, which could not be met by the publishers), it was sggested that the A.I.B.S. consider taking over the responsibilities of publication. The Williams and Wilkins Co. offered to sell publishers' rights to A.I.B.S. for $10, with the agreement that the Waverly Press print the Journal, with costs comparable to other printing, for a period of five years. The rights for publication or reprint of earlier issues (approximately 1925-1951) had been given to the Walter Johnson concern of New York; this did not include extra back copies or reprint rights from 1951 on.

After lengthy discussion, it was moved and passed that A.I.B.S. accept Williams and Wilkins' offer, effective 1957, and that the A.I.B.S. Governing Board delegate authority to the A.I.B.S. Editorial Board, subject to the approval of the A.I.B.S. Executive Board, to appoint editors for five-year terms, beginning 1958.

2. *Translation of Russian Periodicals.* Since N.S.F. has supported translation into English of Russian chemical, mathematical, and physical publications, Director Cox explored the possibility of their support for biological publications. After checking the legal aspects of such activities, it was deemed desirable to have the work done on a contract basis by the Consultants Bureau of New York. After consideration of the pros and cons by the Board Members, it was moved and passed that the A.I.B.S. seek funds from N.S.F. for translating three Russian journals (Microbiology, Plant Physiology, and the Biological and Botanical Sections of the Proceedings of the Academy of Sciences of the U.S.S.R.) on a one-year trial basis. Dr. Cox indicated costs of $15 per page, which included translating, printing, and binding of approximately 1,000 copies. A.I.B.S. would then make these copies available to libraries or individuals.

3. *Other activities.* Group insurance, as presented in the Executive Director's report, was discussed. Suggestions as to a Biological Professional Calendar were received and discussed.

A representative from the Office of Naval Research (O.N.R.), a guest of the Board, talked about the great need for intercommunication between biologists. He suggested that the Board consider ways and means of increasing the exchange of written communication, as well as personnel. A motion was passed to set up an ad hoc committee to study this general problem and present definite suggestions to the International Exchange of Biological Information and Personnel.

The possible cooperation of A.I.B.S. with the Federation of American Societies for Experimental Biology, in undertaking a joint public relations effort to keep the public informed of biological findings, was outlined by President Glass. He stated that a committee had just been appointed from A.I.B.S. and was to begin immediately to explore the possibility of a joint effort by the two biological groups, A.I.B.S. and the Federation of American Societies for Experimental Biology. Representatives from N.S.F. and O.N.R. emphasized the lack of publicity in relation to biological activity, and the rather concerted efforts by the chemical, medical, and physical scientific groups. Some further discussion suggested that unless the public was informed, this would adversely affect federal, state, and other appropriations for biology in the future.

Relationship of A.I.B.S. to member and affiliated societies. Considerable discussion pertaining to this general problem was undertaken by the Board. It was this observer's feeling that A.I.B.S.'s objective was to become a representative for all biologists, by serving them in a manner somewhat similar to the American Chemical Society's method of serving chemists. Some of the Board felt that most biologists, belonging to member and affiliate societies, did not have an understanding of what A.I.B.S. was currently doing, or hoped to do in the future. It was requested that Executive Director Cox prepare material explaining A.I.B.S. activities, and distribute same to member and affiliate societies and others which might possibly be interested in A.I.B.S.'s activities.

Dr. Taylor, a guest representing the American Association for the Advancement of Science (A.A.A.S.), pointed out the anticipated membership changes. It was moved and passed by the Board that A.I.B.S. become an affiliate, instead of an associate, member of A.A.A.S.

Site of 1959 meetings. Because of the scheduling of the International Botanical Conference the last two weeks of August, 1959, in Canada, some difficulty in choosing a site and time for the 1959 meeting of A.I.B.S. was anticipated. The Board suggested meeting in the southwestern part of the United States or on the East Coast prior to or after the International Conference. No need for formal action was indicated by President Glass, since the Executive Director desired only general viewpoints, to serve as a guide for future action.

Nominations. Nominations were made and approved as follows:

President: W. O. Fenn
Vice-President: P. J. Kramer
Secretary-Treasurer: F. P. Cullinan
Executive Committee Member: C. P. Swanson

With the exception of the Secretary-Treasurer, who was to take office immediately, the officers would begin serving January 1, 1957.

OFFICERS, COMMITTEES, AND REPRESENTATIVES OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

OFFICERS

President.....D. V. JOSEPHSON, The Pennsylvania State Univ., University Park
Vice-President.....K. L. TURK, Cornell Univ., Ithaca, N. Y.
Secretary-Treasurer.....H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y.
Journal Editor-in-Chief.....E. O. HERREID, Univ. of Illinois, Urbana

DIRECTORS

C. F. HUFFMAN, Michigan State Univ., East Lansing
(Immediate Past President)

E. L. FOUTS, Univ. of Florida, Gainesville (1958)
I. W. RUPEL, Texas A & M College, College Station (1958)
G. M. WERNER, Univ. of Wisconsin, Madison (1959)
E. H. PARFITT, Evaporated Milk Association, 228 N. LaSalle St., Chicago (1959)
E. L. JACK, Univ. of California, Davis (1960)
H. A. HERMAN, National Association of Artificial Breeders, Columbia, Mo. (1960)

STANDING COMMITTEES

Policy

W. V. PRICE, *Chairman*, Univ. of Wisconsin, Madison, Wis. (1960)

I. A. GOULD, The Ohio State Univ., Columbus (1960)
F. J. ARNOLD, Iowa State College, Ames (1959)
J. H. ERB, The Borden Company, Columbus, Ohio (1959)
R. E. HODGSON, USDA, Beltsville, Md. (1959)
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

Auditing

W. H. LIST, *Chairman*, Association of Ice Cream Manufacturers,
405 Lexington Ave., New York 17, N. Y.

RANDALL WHITAKER, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
C. B. BENDER, The Sperry Corporation, 30 Rockefeller Plaza, New York 20, N. Y.

Ballot Tabulating

G. A. BOWLING, *Chairman*, Strathglass Farm,
Port Chester, N. Y.

ALEX C. HERRO, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
H. W. HOWARD, The Borden Company, 350 Madison Ave., New York, N. Y.

Historian

G. M. TROUT, Michigan State Univ., East Lansing

Journal Management

W. M. ROBERTS, *Chairman*, North Carolina State College, Raleigh

D. H. JACOBSEN, 20 N. Wacker Dr., Chicago, Ill.
J. T. REID, Cornell Univ., Ithaca, N. Y.
E. O. HERREID, Univ. of Illinois, Urbana (ex officio)
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

Membership

J. B. FRYE, JR., *Chairman,* Louisiana State Univ., Baton Rouge

W. M. SNYDER, *Associate Chairman,* Student Affiliate Membership, Michigan State Univ., East Lansing

W. S. ARBUCKLE, *Chairman,* Eastern Division, Univ. of Maryland, College Park
L. R. DOWD, *Associate Chairman,* Eastern Division, Univ. of Connecticut, Storrs
H. E. CALBERT, *Chairman,* Central Division, Univ. of Wisconsin, Madison
E. L. THOMAS, *Associate Chairman,* Central Division, Univ. of Minnesota, St. Paul
L. H. RICH, *Chairman,* Rocky Mountain Region, Utah State Univ., Logan
GEORGE STODDARD, *Associate Chairman,* Rocky Mountain Region, Utah State Univ., Logan
H. A. BENDIXEN, *Chairman,* Western Region, State College of Washington, Pullman
F. B. WOLBERG, *Associate Chairman,* Western Division, Oregon State College, Corvallis
P. M. REAVES, *Chairman,* Southern Division, Virginia Polytechnic Institute, Blacksburg
J. E. JOHNSTON, *Associate Chairman,* Southern Division, Louisiana State Univ., Baton Rouge
R. R. RIEL, Science Service, Ottawa, Canada
J. E. MOXLEY, McDonald College of McGill Univ., Quebec, Canada
D. L. GIBSON, Univ. of Saskatchewan, Saskatoon, Canada

Necrology

T. B. HARRISON, *Chairman,* Univ. of Tennessee, Knoxville

K. M. AUTREY, Alabama Polytechnic Institute, Auburn
P. L. KELLY, Univ. of Nebraska, Lincoln
D. J. HANKINSON, Univ. of Massachusetts, Amherst
N. L. VANDEMARK, Univ. of Illinois, Urbana
G. H. WILSTER, Oregon State College, Corvallis
J. J. WILLINGHAM, Texas Technological College, Lubbock

Public Relations

F. J. GREINER, *Chairman,* Ohio Dairy Products Association, 5 E. Long St., Columbus 15, Ohio (1959)

J. HOFFMAN ERB, The Borden Company, 165 N. Washington Ave., Columbus, O. (1958)
R. H. RUMLER, The Holstein-Friesian Association, Brattleboro, Vt. (1958)
P. E. NEWMAN, Beacon Milling Company, Cayuga, N. Y. (1959)
R. B. MAXCY, The Diversey Corporation, Chicago, Ill. (1960)
W. L. DUNKLEY, Univ. of California, Davis (1960)

Nominating

F. J. ARNOLD, *Chairman,* Iowa State College, Ames

C. F. HUFFMAN, Michigan State Univ., East Lansing
O. F. GARRETT, M & R Dietetic Laboratories, 625 Cleveland Ave., Columbus, O.
L. L. RUSOFF, Louisiana State Univ., Baton Rouge
A. J. MORRIS, Utah State Agricultural College, Logan

Program

N. P. RALSTON, *Chairman,* Michigan State Univ., East Lansing

F. J. BABEL, Purdue Univ., West Lafayette, Ind.
S. P. MARSHALL, Univ. of Florida, Gainesville
L. R. FRYMAN, Univ. of Illinois, Urbana
J. W. POU, North Carolina State College, Raleigh
C. F. HUFFMAN, Michigan State Univ., East Lansing

Resolutions

D. M. SEATH, *Chairman*, Univ. of Kentucky, Lexington

R. E. LEIGHTON, Texas A & M College, College Station
C. H. PARSONS, Univ. of Massachusetts, Amherst
J. C. FLAKE, Evaporated Milk Association, Chicago, Ill.
E. B. OBERG, Carnation Company, Los Angeles, Calif.

Student Affiliate

W. W. SNYDER, *Chairman*, Michigan State Univ., East Lansing (1960)

J. E. JOHNSTON, Louisiana State Univ., Baton Rouge (1958)
L. R. DOWD, Univ. of Connecticut, Storrs (1958)
W. L. SLATTER, The Ohio State Univ., Columbus (1959)
P. M. REAVES, Virginia Polytechnic Institute, Blacksburg (1959)
F. G. WARREN, North Carolina State College, Raleigh (1960)

Public Health

L. A. BLACK, *Chairman*, U. S. Public Health Service,
4676 Columbia Parkway, Cincinnati 26, Ohio

A. C. DAHLBERG, Cornell Univ., Ithaca, N. Y.
W. D. POUNDEN, Ohio Agricultural Experiment Station, Wooster
W. A. KRIENKE, Univ. of Florida, Gainesville
E. W. BIRD, Iowa State College, Ames
C. F. HUFFMAN, Michigan State Univ., East Lansing
E. B. KELLOGG, Milk Industry Foundation, Washington 6, D. C.
B. M. ZAKARIASEN, Land O'Lakes Creameries, Inc., Minneapolis 13, Minn.
(Additional industry and public health members to be added)

Divisions

D. J. HANKINSON, *Chairman*, Univ. of Massachusetts, Amherst

K. M. AUTREY, Alabama Polytechnic Institute, Auburn
L. H. RICH, Utah State Agricultural College, Logan
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

AWARD COMMITTEES

American Feed Manufacturers Award

J. C. SHAW, *Chairman*, Univ. of Maryland, College Park (1959)

S. R. SKAGGS, New Mexico College of Agriculture, State College (1958)
G. H. WISE, North Carolina State College, Raleigh (1958)
N. N. ALLEN, Univ. of Wisconsin, Madison (1959)
W. A. COWAN, Univ. of Connecticut, Storrs (1960)
J. F. SYKES, USDA, Bureau of Dairy Industry, Beltsville, Md. (1960)

Borden Award—Manufacturing

F. E. NELSON, *Chairman*, Iowa State College, Ames (1958)

S. T. COULTER, Univ. of Minnesota, St. Paul (1958)
E. O. HERREID, Univ. of Illinois, Urbana (1959)
G. H. HARTMAN, White House Milk Company, Manitowoc, Wis. (1959)
STUART PATTON, The Pennsylvania State Univ., University Park (1960)
BURDET HEINEMANN, Producers Creamery Company, Springfield, Mo. (1960)

Borden Award—Production

G. W. SALISBURY, *Chairman,* Univ. of Illinois, Urbana (1958)

C. W. DUNCAN, Michigan State Univ., East Lansing (1958)
H. E. BECHTEL, Larro Research Farm, Indianola, Iowa (1959)
H. D. EATON, Univ. of Connecticut, Storrs (1959)
I. R. JONES, Oregon State College, Corvallis (1960)
J. F. CAVANAUGH, American Jersey Cattle Club, Columbus, Ohio (1960)

DeLaval Extension Award

L. H. STINNETT, *Chairman,* Oklahoma State Univ., Stillwater (1958)

GEORGE HYATT, JR., North Carolina State College, Raleigh (1958)
F. J. ARNOLD, Iowa State College, Ames (1959)
E. C. SCHEIDENHELM, Univ. of Kentucky, Lexington (1959)
C. W. REAVES, Univ. of Florida, Gainesville (1960)
H. R. SEARLES, Univ. of Minnesota, St. Paul (1960)

Honors

L. A. MOORE, *Chairman,* USDA, Agricultural Research Service, Beltsville, Md.

I. A. GOULD, Ohio State Univ., Columbus
C. F. HUFFMAN, Michigan State Univ., East Lansing

The Dairy Science Teaching Award

N. P. TARASSUK, *Chairman,* Univ. of California, Davis (1958)

C. E. WYLIE, Univ. of Tennessee, Knoxville (1958)
J. T. REID, Cornell Univ., Ithaca (1959)
F. H. HERZER, Mississippi State College, State College (1959)
G. M. TROUT, Michigan State Univ., East Lansing (1960)
C. L. NORTON, Oklahoma State Univ., Stillwater (1960)

SPECIAL COMMITTEES

Education

W. L. SLATTER, *Chairman,* Ohio State Univ., Columbus

G. H. WISE, North Carolina State College, Raleigh
H. L. MITTEN, JR., 574 Wilshire Ave., Glen Ellyn, Ill.
C. C. OLSON, North Dakota State Agricultural College, Fargo
J. T. LAZAR, JR., Clemson Agricultural College, Clemson, S. C.
E. L. JACK, Univ. of California, Davis (ex officio)

Personnel Manual Preparation

H. B. HENDERSON, *Chairman,* Univ. of Georgia, Athens

I. A. GOULD, The Ohio State Univ., Columbus
H. J. WILLIAMS, Carnation Company, Los Angeles, Calif.
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y.
F. H. GRAY, Southern Dairies, Inc., Charlotte, N. C.
F. C. EWBANK, Michigan Milk Producers' Assn., 355 East Second Street, Imlay City, Mich.

Association Representatives

American Association for the Advancement of Science: J. W. THOMAS, Bureau of Dairy Industry, USDA, Beltsville, Md.

Committee on Inter-Society Cooperation: L. A. MOORE, Dairy Husbandry Research Branch, USDA, Beltsville, Md.

Dairy Remembrance Fund: D. V. JOSEPHSON, *Chairman,* The Pennsylvania State Univ., University Park; J. C. SHAW, Univ. of Maryland, College Park; A. C. DAHLBERG, Cornell Univ., Ithaca, N. Y.

Dairy-wide Coordinating Committee on Nutrition Research: H. F. JUDKINS, *Chairman,* 32 Ridgeway Circle, White Plains, N. Y.; J. D. INGLE, 9825 S. Peoria Street, Chicago, Ill.; G. C. NORTH, Beatrice Foods Company, 1526 S. State Street, Chicago, Ill.

National Research Council: W. E. KRAUSS, Ohio Agricultural Experiment Station, Wooster

Ralston-Purina Research Fellowship Committee: J. B. FRYE, JR., Louisiana State Univ., Baton Rouge

The Sub-Committee on Education and Training of the Dairy Industry Committee Representative: H. B. HENDERSON, Univ. of Georgia, Athens

American Institute of Biological Sciences Representative: D. L. HILL, Purdue Univ., West Lafayette, Ind.

3-A Sanitary Standards Committee: J. R. BRUNNER, Michigan State Univ., East Lansing

U. S. Livestock Sanitary Association: W. D. KNOX, *Hoard's Dairyman,* Fort Atkinson, Wis.

National Association of Artificial Breeders: J. O. ALMQUIST, The Pennsylvania State Univ., University Park

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B. H. WEBB, *Vice-Chairman,* National Dairy Research Laboratories, Oakdale, L. I., N. Y.
STUART PATTON, *Secretary,* The Pennsylvania State Univ., University Park

SECTION COMMITTEES

Program

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B. H. WEBB, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
STUART PATTON, The Pennsylvania State Univ., University Park

Butter

T. R. FREEMAN, *Chairman,* Univ. of Kentucky, Lexington
E. W. BIRD, Iowa State College, Ames
H. J. BUYENS, 1436 North Lawler, Chicago, Ill.
CLAUDE HARPER, JR., Beatrice Foods Co., 1526 S. State St., Chicago 5, Ill.
S. R. HOOVER, Eastern Regional Research Laboratory, Philadelphia, Pa.
CHRIS JENSEN, North Dakota Agricultural College, Fargo

Nomenclature and Methodology of Milk Proteins

B. L. LARSON, *Chairman,* Univ. of Illinois, Urbana
J. R. BRUNNER, Michigan State Univ., East Lansing
ROBERT JENNESS, Univ. of Minnesota, St. Paul
N. P. TARASSUK, Univ. of California, Davis
C. H. WHITNAH, Kansas State College, Manhattan
R. M. WHITNEY, Univ. of Illinois, Urbana
C. A. ZITTLE, Eastern Utilization Research Branch, 600 E. Mermaid Lane, Philadelphia 18, Pa.

Dairy Products Judging

G. M. TROUT, *Chairman,* Michigan State Univ., East Lansing
C. J. BABCOCK, USDA, Washington, D. C.

L. R. DOWD, Univ. of Connecticut, Storrs
P. A. DOWNS, Univ. of Nebraska, Lincoln
E. L. THOMAS, Univ. of Minnesota, St. Paul
F. C. WARREN, North Carolina State College, Raleigh
J. J. WILLINGHAM, Texas Technological College, Lubbock

Sub-Committee to Develop a Score-Card for Nonfat Dry Milk

E. L. THOMAS, *Chairman*, Univ. of Minnesota, St. Paul
F. E. FENTON, USDA, Washington, D. C.
RAY POWERS, The Borden Company, New York, N. Y.
R. P. CHOI, American Dry Milk Institute, 221 N. LaSalle St., Chicago, Ill.
W. K. STONE, Virginia Polytechnic Institute, Blacksburg
N. P. TARASSUK, Univ. of California, Davis
B. M. ZAKARIASEN, Land O'Lakes Creameries, Inc., Minneapolis 13, Minn.

Curd Tension of Milk

L. K. CROWE, *Chairman*, Univ. of Nebraska, Lincoln
U. S. ASHWORTH, State College of Washington, Pullman
L. W. AURAND, North Carolina State College, Raleigh
J. H. HETRICK, Dean Milk Co., 1126 Kilborn Ave., Rockford, Ill.
H. A. RISHOI, Cherry Burrell Corp., 426 W. Randolph St., Chicago, Ill.

Resolutions

J. C. FLAKE, *Chairman*, 325 Callan St., Evanston, Ill.
V. H. NIELSEN, Iowa State College, Ames
C. E. PARMELEE, Purdue Univ., West Lafayette, Ind.

Nominations

C. L. HANKINSON, *Chairman*, Ohio State Univ., Columbus
H. E. CALBERT, Univ. of Wisconsin, Madison
O. F. GARRETT, M & R Dietetic Laboratories, 625 Cleveland Ave., Columbus, O.

OFFICERS AND COMMITTEES OF THE PRODUCTION SECTI

SECTION OFFICERS

S. P. MARSHALL, *Chairman*, Univ. of Florida, Gainesville
N. L. JACOBSON, *Vice-Chairman*, Iowa State College, Ames
G. W. TRIMBERGER, *Secretary*, Cornell Univ., Ithaca, N. Y.

SECTION COMMITTEES

Dairy Cattle Judging

H. E. KAESER, *Chairman*, The Ohio State Univ., Columbus (1958)
W. R. MURLEY, North Carolina State College, Raleigh (1959)
G. B. MARION, Kansas State College, Manhattan (1960)

Guidance in Conduct Production Section Session

R. E. ERB, *Chairman*, State College of Washington, Pullman (1958)
N. P. RALSTON, Michigan State Univ., East Lansing (1959)
S. W. MEAD, Univ. of California, Davis (1960)

Program

S. P. MARSHALL, *Chairman,* Univ. of Florida, Gainesville
N. L. JACOBSON, Iowa State College, Ames
G. W. TRIMBERGER, Cornell Univ., Ithaca, N. Y.

Resolutions

R. E. LEIGHTON, *Chairman,* Texas A & M College, College Station
A. E. DRACY, South Dakota State College, College Station
R. E. MATHER, Rutgers Univ., New Brunswick, N. J.
D. J. HILL, Purdue Univ., West Lafayette, Ind.

JOINT COMMITTEES OF THE PRODUCTION AND EXTENSION SECTIONS

EXTENSION — PRODUCTION

Breed Relations

(Chairman in Production each year)

RALPH WAYNE, Univ. of Minnesota, St. Paul (1958)
C. H. BOYNTON, Univ. of New Hampshire, Durham (1959)
G. E. GORDON, Univ. of California, Berkeley (1960)

K. A. HUSTON, *Chairman,* Kansas State College, Manhattan (1958)
C. F. FOREMAN, Iowa State College, Ames (1959)
H. W. THOELE, Pennsylvania State Univ., University Park (1960)

Dairy Cattle Breeding

(Chairman in Extension each year)

S. N. GAUNT, *Chairman,* Univ. of Massachusetts, Amherst (1958)
H. W. CARTER, Cornell Univ., Ithaca, N. Y. (1959)
C. D. MCGREW, Ohio State Univ., Columbus (1960)

L. D. MCGILLIARD, Univ. of Michigan, East Lansing (1958)
R. C. LABEN, Univ. of California, Davis (1959)
L. O. GILMORE, Ohio State Univ., Columbus (1960)

Type

(Chairman alternates. From Production in 1958)

J. G. CASH, Univ. of Illinois, Urbana (1958)
RAY ALBRECTSEN, Cornell Univ., Ithaca, N. Y. (1959)
CLYDE CHAPPELL, Univ. of Tennessee, Knoxville (1960)

J. W. POU, *Chairman,* North Carolina State College, Raleigh (1958)
F. W. ATKESON, Kansas State College, Manhattan (1959)
C. L. BRANTON, Louisiana State Univ., Baton Rouge (1960)

Dairy Cattle Health

(Chairman alternates. From Production in 1958)

C. G. BRADT, Cornell Univ., Ithaca, N. Y. (1958)
E. T. ITSCHNER, Univ. of Missouri, Columbia (1959)
F. H. EWALT, Oregon State College, Corvallis (1960)

J. C. SHAW, *Chairman,* Univ. of Maryland, College Park (1959)
H. J. HILL, Colorado A & M College, Fort Collins (1958)
W. A. KING, Clemson Agricultural College, Clemson, S. C. (1960)

OFFICERS AND COMMITTEES OF THE EXTENSION SECTION
1957–1958

SECTION OFFICERS

L. R. FRYMAN, *Chairman,* Univ. of Illinois, Urbana
L. A. JOHNSON, *Vice-Chairman,* Michigan State Univ., East Lansing
C. W. NIBLER, *Secretary,* Univ. of Nebraska, Lincoln

SECTION COMMITTEES

Dairy Records

D. E. VOELKER, *Chairman,* Iowa State College, Ames (1959)
J. W. CROWLEY, Univ. of Wisconsin, Madison (1958)
H. C. GILMORE, Pennsylvania State Univ., University Park (1958)
MARVIN SENGER, North Carolina State College, Raleigh (1960)
E. R. BONEWITZ, Kansas State College, Manhattan (1960)

Resolutions

CLARENCE PARSONS, *Chairman,* Massachusetts State Univ., Amherst (1959)
C. W. NIBLER, Univ. of Nebraska, Lincoln (1958)
W. R. VAN SANT, Univ. of Arizona, Tucson (1960)

Teaching Methods

J. D. GEORGE, *Chairman,* North Carolina State College, Raleigh (1960)
L. H. STINNETT, Oklahoma State Univ., Stillwater (1959)
M. F. ELLMORE, Virginia Polytechnic Institute, Blacksburg (1959)
W. R. HESSELTINE, Connecticut State Univ., Storrs (1958)

4-H Club

W. N. PATTERSON, *Chairman,* Virginia Polytechnic Institute, Blacksburg (1958)
W. P. ANDERSON, Pennsylvania State Univ., University Park (1959)
A. M. MEEKMAN, A & M College of Texas, College Station (1960)
C. C. OLSON, North Dakota State College, Fargo (1960)

ABSTRACTS OF PAPERS

PRESENTED AT THE WESTERN DIVISION MEETING

PULLMAN, WASHINGTON, JULY 14-17, 1957

A Method for Selecting Drying Conditions for the Determination of the Total Solids in Milk. U. S. ASHWORTH, State College of Washington, Pullman.

A standard milk may be prepared accurately from spray-dried powder of known composition. This milk can then be used to verify the drying rate of an oven which is being used for total solids determinations of fresh or concentrated milks. A curve may be prepared by plotting the calculated per cent total solids remaining after any drying time in the oven against time. The point where this curve crosses the known value for total solids in the standard milk solution gives the optimum drying time for the conditions being employed. The standard milk has special value in checking any of the recently developed rapid drying methods for total solids in milk.

Factors Affecting the Dye Binding of Milk Proteins. U. S. ASHWORTH AND R. SEALS, State College of Washington, Pullman.

A rapid and simple procedure for the estimation of protein in milk is based on the reaction between the acid dye, Orange G, and the basic groups of the milk proteins. The only reagent needed is one containing 0.1% of the dye and 2.1% of citric acid. Fresh milk or powder containing 20-50 mg. of protein was shaken with 25 ml. of the dye and allowed to stand for about 1 hr. The protein-dye complex was then removed either by filtering or by centrifuging. The optical density of the clear filtrate was read at 420 mμ. A special flow-through light absorption cell is described. This cuvette has a 1-mm. length light-path which made further dilution of the dye unnecessary.

The error due to absorption of dye by the filter paper is small and compensated for by filtering the standard. In the temperature range between 46 and 130° F. no significant effect of temperature on dye-binding could be observed. Whey proteins gave higher binding values (mg. dye per gram of protein) than did casein. Alpha-casein bound more dye than beta-casein. The dialyzable nitrogen fraction bound no dye.

Colorimetric Determination of Lactose in Milk Using the Phenol-Sulfuric Acid Reaction. D. R. ERICKSON AND G. A. RICHARDSON, Oregon State College, Corvallis.

A rapid and simple colorimetric method for the determination of lactose in milk is described which gives results in agreement with the official polarimetric method. It consists of mixing 10 ml. of milk diluted with 100 ml. of water, with 7 ml. of 5% dialyzed iron, bringing to 250 ml. volume with distilled water, and filtering. The resulting clear filtrate is 1 to 100, and 2 ml. of this was transferred to a 19 by 105 mm. cuvette. One ml. of a 5% phenol solution was added to the cuvette, followed by 5 ml. of concentrated H_2SO_4 C. P. The developed color was read in a spectrophotometer at 490 mμ. The color was stable and followed the Beer-Lampert law. Data showed that the method gave results in statistical agreement with the official polarimetric method, when the technique, especially the rate of addition of the sulfuric acid, is standardized.

Plastics for Hydrometry. N. S. GOLDING, Professor Emeritus, State College of Washington, Pullman.

The objective of this study has been to develop special types of plastic lactometers, to determine the density of whole milk, skimmilk, and whey.

The conventional glass lactometers have two disadvantages:

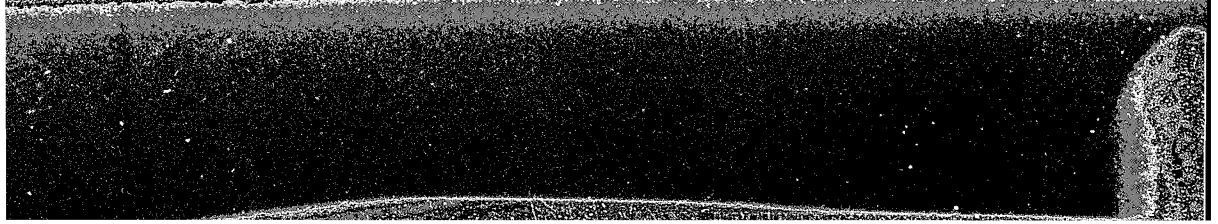
1. They are fragile and the more accurate they are made the more fragile they become; a larger sample of milk must then be used.
2. The coefficient of thermal expansion of glass is many times less than water, making accurate temperature control essential.

Several plastics lower in density than most wheys do not have these disadvantages. These plastics have been studied for their suitability for hydrometers and most of them have one or more desirable properties. However, polymethylstyrene (CYMAC-400) thus far has all the physical properties necessary for making an accurate hydrometer.

When CYMAC-400 is mixed cold and then mixed hot with very fine barium sulphate and a small amount of insoluble color, pressure-injection molded discs or spheres of any required density have been made by varying the percentage of barium sulphate. The advantage of this plastic is that it has the same thermal expansion as water at 20 to 25° C. Annealed discs of CYMAC-400 maintained their exact density in milk when changed and washed weekly for 4 mo.

Panels as a Means of Determining Consumer Acceptance of Dairy Products. R. R. HEDRICK, J. L. BRENCE, AND J. A. NELSON, Dairy Industry Department, Montana State College, Bozeman.

Panels have been used to a limited extent in determining consumer acceptance of dairy products, and more extensively to determine consumer preferences of other foods. In se-



lecting the panel, experience, availability, age, sex, health, taste and smell sensitivity, reliability of judgment, and other psychological factors have been taken into consideration. The size of the panel has been governed by availability and cost; the larger the panel, the more reliable the results, but a panel with a small number of members with ability to taste is considered more conducive to accuracy than a large one with low taste abilities. Importance has been placed on the management of the panel, condition and manner in which the samples are presented to the panel members, and the condition of the judging room. For more accurate results, panels have been organized so that the data can be analyzed statistically.

Paper Electrophoresis of Casein. L. M. Libbey and U. S. Ashworth, State College of Washington, Pullman.

A study of the paper electrophoresis of casein was made with buffers of about pH 12. A KOH-K_2HPO_4 buffer of pH 12.3 and ionic strength of 0.19 clearly resolves casein into three fractions, in the Spinco apparatus run at 20-40 milliamperes for 30 hr., preferably at about 4° C. The level of sample application was critical; 0.01—0.02 ml. of an approximately 10% protein whole casein sol is recommended. To achieve the highest degree of resolution, the sample was placed as near as possible to the cathode by cutting a special slot in the Durrum type cell. After electrophoresis, the paper strips were dyed with bromphenol blue. Without correcting for tailing errors, the relative distribution of the casein fractions, based on areas beneath scanning curves, was approximately 50% alpha- and kappa-, 45% beta-, and 5% gamma-casein.

Effect of Ingredients of Chocolate Drink on Consumer Acceptance. H. S. Willard, W. R. Thomas, and W. D. Schutz, University of Wyoming, Laramie.

On the basis of chi-square values, a panel of 40 or more students showed a definite preference for 1% fat over no fat, 3% fat over 1% fat, 11% solids-not-fat over 9% solids-not-fat, vanillin flavoring over pure vanilla flavoring, 7% sugar over 3% sugar, and 1% over 1.5% Dutch Process cocoa. The students were about evenly divided on preference of high- and low-viscosity chocolate drinks. The panel used both the hedonic scale and the rank-order method of expressing preference of three samples at each taste session. The rank-order scale method proved to be more sensitive than the hedonic scale method, but the latter brought out the degree of dislikes and likes. The basic mix in each test consisted of 93.98% milk testing 2% butterfat, 1.24% Dutch Process cocoa, 4.75% beet sugar, and 0.03% stabilier. Improvement in score and a decrease in the percentage dislikes resulted by adding 0.7% vanillin and 7% beet sugar, and decreasing cocoa to 1%.

Determination of the Fat Content of Cottage Cheese with Modified Babcock Methods. E. A. Zottola and R. W. Stein, Oregon State College, Corvallis.

Four series of tests were run, to find a satisfactory method for testing creamed cottage cheese for fat. In three of the series, tests were made on pints of creamed cottage cheese obtained from local grocery stores; the fourth series was run on cottage cheese creamed in the laboratory. Five methods were studied: *(1)* ether extraction, *(2)* D.P.S. detergent, *(3)* butyl alcohol, *(4)* Minnesota modification, and *(5)* Pennsylvania modification. In this study, the ether extraction method was assumed to give correct results and was the standard. The data showed that of the four methods, only the butyl alcohol method compared favorably with the ether extraction method. On laboratory-perpared samples, the butyl alcohol test showed lower fat readings than the ether extraction method, except in products containing more than 5% fat, in which the reverse was true.

Effects of Adding Seminal Plasma to Concentrated Sperm Samples at 37° C. J. L. Albright, M. H. Ehlers, and R. E. Erb, State College of Washington, Pullman.

Seminal plasma is used as a vehicle for spermatozoa and supplies energy for their metabolism. A study was conducted to determine whether washed sperm-citrate samples, devoid of extracellular nutrients and showing marked declines in motility, could utilize plasma nutrients.

By centrifugation, sperm from 23 samples were removed and twice-washed with sodium citrate buffer. Restoration to original plasma volume was with sodium citrate and is referred to as semen-equivalent. The 0.2 ml. semen-equivalent plus 0.8 ml. citrate samples was placed in a 37° C. water bath. Progressive motility estimates were recorded initially and at hourly intervals for 4 hr. before and after the addition of 0.1 and 0.2 ml. of seminal plasma.

Results were as follows:

	Hours postincubation				
	Initial	1	2	3	4
Before adding 0.1 plasma	24	6	1	0	0
After adding 0.1 plasma	—	26	20	13	3
After adding 0.2 plasma	—	40	38	20	23
Excess, adding 0.8 ml. plasma	65	56	48	39	33

Concentrated sperm-citrate samples that showed little or no progressive motility and appeared to be dead, could be restimulated by adding nutrients in seminal plasma.

Alternate Five-Day Injections of Oxytocin as a Means of Maintaining Maximum Milk Production. D. V. ARMSTRONG AND G. M. WARD, Department of Dairy Industry, Colorado State University, Fort Collins.

This study was initiated to determine whether intramuscular injections of oxytocin would maintain production in hard-milking cows which showed a low degree of persistency. The effect upon milking rate was also investigated. Five hard-milking cows were selected: two Brown Swiss, two Holsteins, and one Guernsey, and injected intramuscularly with either 30 or 40 U.S.P. units of oxytocin immediately before milking. The injections were continued for five days and followed by a five-day control period, after which this procedure was repeated. The study covered from 100 to 130 days.

The average increase in daily milk production for the period of injection over the control period for the five cows was 1.6, 0.7, 1.6, 1.2, and 4.3 lb. The average milking time for all of the cows was reduced from 8.4 min. during the control period to 6.5 min. during the periods of injection. The injections had no effect upon the regularity of heat periods.

Performance of Lactating Dairy Cows and Carrying Capacity of Pastures as Influenced by Supplemental Feeding and Seasonal Pasture Growth. C. W. ARAVE, C. H. MICKELSON, G. E. STODDARD, AND G. Q. BATEMAN, Utah State University, Logan.

During the summer, pasture forage from an improved seeding was clipped and fed to four groups of Holsteins. They were fed individually all the clipped pasturage they would eat and, in addition, Group 1 received 1 lb. of grain for each 5 lb. of milk; Group 2 received 1 lb. of grain for each 10 lb. of milk; Group 3 received no grain and hay; and Group 4 received 10 lb. of hay daily, of which 9.1 was consumed.

Groups 1 to 4 consumed 1,010, 420, 0, and 0 lb. of grain, respectively, during 122 days. Group 4 consumed 1,106 lb. of hay. Cows in Groups 1, 2, 3, and 4 produced 4,274, 3,689, 3,881, and 4,144 lb., respectively, of 4% FCM which, when corrected for differences in production during a ten-day preliminary period, amounted to 4,274, 4,465, 3,661, and 4,323 lb. of 4% FCM. There were no measurable differences in body weight, and all groups increased in weight. Yields of dry matter for silage or hay were comparable.

Effect of Continuous Feeding of Slaked Lime and Vitamin A to Calves on a Diet of Reconstituted Whey, Grain, and Hay to 60 Days of Age. D. C. BROWN, H. S. WILLARD, AND J. W. HAMILTON, University of Wyoming, Laramie.

Two series of experiments with 28 and 35 Holstein calves, respectively, in each series, were conducted to determine if the incidence of intestinal irritation and dry, scaly skin could be reduced when slaked lime was fed to prevent scour. When the liquid part of the ration was reduced from 10 to 6 lb. daily, from 30 to 60 days of age, there was no indication that slaked lime acted as an intestinal irritant. Scaly skin was noted in all whey-fed calves, but to a lesser extent in calves which started to eat grain at an early age. The scaly skin condition disappeared soon after the calves began eating from ¾-lb. to 1 lb. of grain daily. Sixteen calves fed a capsule containing 12,000 units of vitamin A in 4 ml. of pure cottonseed oil each day between 20 and 60 days of age, had sleeker coats than the controls, but the group receiving the vitamin A had scours more often and their growth rate was less than the control group. The authors believe that the increase in rate of scouring of the vitamin A–fed calves was due to the cottonseed oil carrier. Statistical analysis of the data indicated that sex of calf was not related to treatment.

Relationship of Seminal Fructose and Free Amino Acids Concentration to Sexual Maturation of the Bull. F. X. GASSNER AND M. L. HOPWOOD, Colorado State University, Fort Collins.

In studies previously reported, it was shown that seminal free amino acids rise in the adolescent bull and reach the adult pattern at about 20 mo., then remain plateaued to at least 12 yr. of age.

It was also shown that seminal fructose was present at a near-mature level at 11 mo. of age, indicating that the Leydig Cell system begins to function before spermatogenesis occurs.

In order to establish at what age seminal fructose appears and reaches the adult level, seminal fluid was collected by electroejaculation from 26 bull calves, 7 to 10 mo. old. Fructose and free seminal amino acids were determined by methods previously reported.

Seminal fructose concentration steadily increased from 27 mg/100 ml. at 7 mo. to 65 mg/100 ml. at 9 mo. of age. At 11 mo., it was 370 mg/100 ml. and at 12 mo., it reached 580 mg/100 ml., which is considered the mature level. Therefore, maturation of the Leydig Cell system, when measured by fructose production, occurred at least 3 mo. before the mature pattern of seminal amino acids and spermatogenesis began to become established.

Free Amino Acid Concentration in Bovine Testes and Accessory Sex Organs. M. L. HOPWOOD AND F. X. GASSNER, Colorado State University, Fort Collins.

The free seminal amino acid pattern of maturing bulls became apparent by the 15th

mo. of age, reaching a plateau at 20 mo. Seminal fluid of the immature bull lacks glutamic acid, which appears in direct proportions to sperm production. While amino acids disappear after castration or vasectomy, all except glutamic acid reappear following testosterone treatment. Since it seems that glutamic acid is primarily a testicular product and is also related to spermatogenesis, it became of interest to trace its origin.

Testes, epididymides, ampullae, and seminal vesicles from ten normal bulls were obtained and, after expressing fluid from all but the testes, were frozen until used. Ethanol extracts were chromatographed on paper to determine the amino acids.

Testes showed the highest concentration of glutamic acid. Fluid from epididymides was most concentrated. Fluids and tissues of the ampullae and seminal vesicles, however, were richer in alanine. This indicates that the organs producing and storing up to and through maturation elaborate the high concentration of glutamic acid found in the ejaculate. It also is indicated that glutamic acid fulfills a function on which normal development of spermatozoa depends.

Influence of Altering the Amount of Plasma on Semen Metabolism. N. S. KUSHWAHA, M. H. EHLERS, AND R. E. ERB, State College of Washington, Pullman.

Semen glycolysis was studied by varying the amount of seminal plasma. Each of 28 samples from three bulls was divided into three portions: I, whole semen; II, semen with volume reduced to one-half by removal of much of the plasma; and III, semen with volume doubled by added plasma. The citrate-buffered portions were incubated at 37° C., with fructose and lactic acid being determined at 0, ½, and 1 hr. After ½ hr. incubation, fructose utilization based on sperm numbers was about ⅓ higher in I and III than in II. At 1 hr., utilization was highest in III. Lactic acid formation was greatest in III for both incubation intervals.

This experiment emphasizes the twofold problem in selecting an incubation time. An incubation of ½ hr. at 37° C. will underestimate glycolytic rate for samples having a combination of low fructose and high sperm concentration. Samples with high fructose and low sperm concentration, however, may have excessive errors in measurement with an incubation of ½ or 1 hr.

Intermittent Colostrum Feeding and Its Effect upon Blood Plasma, Viatmin A, Carotene, Calcium, and Phosphorus of Dairy Calves. E. A. KEYES, E. J. PEACE, AND J. L. BRENCE, Dairy Industry Department, Montana Agricultural Experiment Station, Bozeman.

Thirty-six Holstein calves were divided at birth and fed in three groups for 80 days. Group I was fed colostrum and whole milk intermittently. Group II was fed colostrum the first four days and Holstein whole milk from different cows intermittently, and Group III was fed colostrum the first four days and Holstein milk continuously from one cow. Colostrum was substituted for milk of equal weight, with no scouring effect due to the colostrum. The calves were weighed and total digestible nutrients were computed on the feed eaten. Blood analyses were made for vitamin A, carotene, calcium, and phosphorus. Samples were taken on the fourth, 16th, and 20th days. There was no visible difference between the calves in Groups I and II or Groups II and III. There was no significant difference between any of the three groups of calves when total digestible nutrients consumed were compared with gain in weight. Blood plasma analyses indicated no significant difference between groups. Growth rates were normal. Results indicate that equal weights of colostrum may be substituted for milk in feeding young dairy calves, without causing any detrimental effects or changing the blood plasma analyses from normal calves.

A Comparison of Conventional and Self-Feeding Dairy Cows for the Honolulu Milk Market. K. K. OTAGAKI AND K. MORITA, University of Hawaii, Honolulu.

Good-quality forages generally supply the most economical source of nutrients, including vitamins, minerals, and proteins. However, the extended use of pasture and cultivated soilage crops in Hawaii is limited, because of the tremendous urbanization of the existing farm areas and the resulting scarcity of desirable agricultural areas for economical forage production.

It might be feasible to increase the concentrates and to feed no more green roughage than is necessary to prevent animals from going off feed and to provide a proper mixture for efficient rumen activity.

Feeding trials with minimum roughage feeding, accompanied by self-feeding of concentrates, showed profitable returns over feed costs, when compared with the conventional twice-a-day feeding. Better milkers have produced at higher levels and have maintained milk production over an extended period. The self-fed lots also showed improvement in general appearance and body weight gains at the termination of the experiment.

Nutritive Value of Molasses-Bagasse Pith Mixtures when Fed to Dairy Heifers. P. A. PUTNAM AND T. H. BLOSSER, State College of Washington, Pullman.

Six yearling Jersey heifers, 4 mo. in gestation, were used to determine the apparent digestibility and TDN contents of 2:1 and 4:1 molasses-bagasse pith mixtures, when fed

with chopped alfalfa hay. After a ten-day preliminary period and a seven-day collection period, during which the digestibility of alfalfa was determined, the heifers were divided into two groups and fed 6 lb. daily of the molasses-bagasse mixtures. Digestibility was determined by difference, using the chromic oxide indicator method and grab samples of feces collected three times daily. The average TDN content of the hay on an as-fed basis was 43.0 (49.7 on a 100% dry basis). The 2:1 molasses-bagasse mixture contained 45.1% TDN (56.2 on a 100% dry basis) and the 4:1 mixture 62.8% TDN (80.3 on a 100% dry basis).

Corn Steep Liquor as a Preservative for Grass Silage. J. R. SCHUBERT, J. V. BATEMAN, I. R. JONES, AND P. H. WESWIG, Oregon State College, Corvallis.

Three one-ton silos were filled with second-cutting alfalfa and grass, one untreated, one with 70 lb/T molasses and one with 100 lb/T corn steep liquor.[1] The untreated silage (pH 5.08) underwent extensive butyric acid deterioration. This was considerably improved by adding molasses (pH 4.62) or corn steep liquor (pH 4.70). The respective nonvolatile/volatile acid ratios were 0.49, 0.92, and 0.72. The silages were fed to pairs of dairy heifers for five-day, total collection digestion trials. Digestion coefficients of 60.1, 60.5, and 66.9 for the dry matter contents were obtained for the untreated (26.3% D.M.), molasses (28.1% D.M.), and corn steep liquor (25.1% D.M.) silages, respectively. Similarly, crude protein contents of 11.6, 11.6, and 14.0%, dry basis, showed 62.6, 60.2, and 74.6 digestion coefficients. T.D.N. values of 58.9, 57.9, and 63.8 were calculated, the latter reflecting improved digestibility of each of the proximate principles of corn steep liquor silage. Corn steep liquor appears to be a potential preservative for grass sligae, based on enhanced feeding value, as well as a nutrient source for microorganisms.

[1] Generously provided by the A. E. Staley Manufacturing Company, Decatur, Illinois. Solids, 50%; pH, 3.9; $N \times 6.25$, 21.9%; lactic acid 9.8% (wet basis).

Comparative Value of Alfalfa Hay and Alfalfa Silage as Winter Roughage for Dairy Cows. G. E. STODDARD, C. H. MICKELSEN, AND C. Q. BATEMAN, Utah State University, Logan.

During 1956, third-crop alfalfa from 16.72 acres was harvested, half as silage and half as hay. Alfalfa for silage was chopped directly from the stump and ensiled in an upright concrete stave silo, half without preservative and half with sodium metabisulfite, at the rate of 8 lb. per ton of green weight. Both hay and silage were of very good quality. Forage fed was 2,504 lb. of air dry matter for the hay and 2,581 lb. for the silage.

Twelve cows were paired and assigned to one of three groups: Group 1, hay and corn silage; Group 2, hay and alfalfa silage, and Group 3, alfalfa silage and corn silage. Results indicated that dry matter consumption from alfalfa silage was not as great as from either alfalfa hay or corn silage. Milk production was not maintained as well when alfalfa silage replaced either hay or corn silage in the ration. No differences in body weight were observed.

All-Roughage vs. Roughage and Concentrate Feeding for Holstein Steers. R. O. RUPP, G. M. WARD, AND M. H. HAZALEUS, Colorado State University, Fort Collins.

As a part of a larger project, this study was undertaken to determine the effect of adding concentrates to the ration of Holstein steers on *(1)* the returns above feed cost, *(2)* the rate of gain, *(3)* the finish at 1,000 lb., *(4)* the length of time required to reach 1,000 lb., and *(5)* the percentage of wholesale cuts.

Green chopped alfalfa, alfalfa hay, and corn silage were fed and the grain consisted of one-half corn and one-half dried beet pulp. The steers were 1 yr. of age when the feeding trial began.

The grain-fed group was slaughtered 100 days later and the all-roughage group 156 days later. The grain-fed and all-roghage fed steers, respectively, weighed 1,004 and 1,031 lb. at slaughter, gained 2.7 and 1.8 lb. per day, dressed 53.1 and 53.3%, and the returns above feed cost were $14 and $10. The percentage of wholesale cuts and of separable bone, fat, and lean was almost the same for both groups. All of the animals in both groups graded U. S. Standard.

Carotene and Vitamin A Content of Bovine Liver Before and After Parturition. P. H. WESWIG, I. R. JONES, R. G. SPROWLS, AND J. F. BONE, Oregon Agricultural Experiment Station, Corvallis.

The carotene and vitamin A content of livers of 15 normal cows from the station herd was determined from biopsy specimens. Average carotene and vitamin A values were 12.0 γ/g and 129.0 γ/g liver, respectively, 4 wk. before parturition, and 12.6 γ/g and 120.0 γ/g liver 4 wk. postpartum. With carotene intakes ranging from 130 to 390 γ per kilo body weight, twelve livers averaged 1.5 γ/g and 13.6 γ/g liver carotene and vitamin A, respectively, 4 wk. before parturition, and 1.3 γ/g and 12.8 γ/g liver 4 wk. postpartum. Blood and milk fat samples were taken at the time of biopsy. Also, in selected instances, carotene and vitamin A were determined on colostrum.

No significant changes in prepartum and

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postpartum liver contents were noted in either group. Comparisons over periods longer than 2 mo. may reflect seasonal variation in normal animals. Twenty-four parturitions occurred when liver stores were maximum (July-December). It appears that liver stores of carotene and vitamin A do not change significantly during the period 4 wk. before or after parturition.

Effect of Stage of Lactation on the Solid Components of Milk from Individual Holstein-Friesian Cows. W. S. NICHOLSON, JR., W. R. THOMAS, H. S. WILLARD, AND D. C. BROWN, University of Wyoming, Laramie.

This paper describes the variations observed in 10 mo. lactation of 18 cows for 3 yr. Samples were collected monthly from individual cows during two consecutive milkings. Total solids were determined by the Dietert method, fat by the Babcock method, and solids-not-fat by difference. Analysis of variance of the monthly yield of milk, solids-not-fat, and total solids showed significant differences between cows at the 1% level. The differences in monthly yields during lactation were found to be the result of both linear and quadratic regression. Butterfat yields were due solely to regression. Butterfat percentage did not conform statistically either to linear or to quadratic regression. Solids-not-fat and total solids percentage data paralleled closely those for butterfat percentage. However, the level of significance for quadratic regression and for combined linear and quadratic regression was only 5%. The data indicate that it is difficult to fit a smoothed curve from percentages of milk constituents obtained over a lactation period.

APPLICATION OF ELECTROPHORESIS ON PAPER TO THE ESTIMATION OF ALPHA-, BETA-, AND GAMMA-CASEIN

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SUMMARY

Alpha-, beta-, and gamma-components of casein were separated by paper electrophoresis, and the dyed proteins in paper were estimated by direct photometry. Correction factors were determined because the components did not bind dye in proportion to their concentration. Protein concentrations corrected for differences in dye-binding capacities agreed with results of protein-nitrogen analyses, and casein composition was alpha-, 60%; beta-, 32%, and gamma-, 9%.

Mellander *(11)* showed, by means of free electrophoresis, that casein is composed of three electrophoretic fractions. He named these alpha-, beta-, and gamma-casein, in order of decreasing mobilities. Warner *(15)* used the same methods, but was unable to obtain unequivocal evidence of the presence of gamma-casein. He reported the relative amounts of the alpha- and beta-fractions as 80.7 and 19.3%, respectively. Hipp, Groves, Custer, and McMeekin *(7)* isolated gamma-casein by isoelectric precipitation and the use of alcohol. Purity of the gamma-fraction was checked by electrophoresis. They reported the composition of unfractionated casein, determined by electrophoretic analysis, as 75% alpha-casein, 22% beta-casein, and 3% gamma-casein. Similar results have been obtained by Neilsen, Kucera, and Bird *(12)*.

Zweig and Block *(16)* applied electrophoresis in filter paper (zone electrophoresis) to the separation of components of casein. Their method was essentially the same as that used by Durrum *(3)* for the separation of serum proteins. They found the relative mobilities of the same order of magnitude as those which were obtained by the Tiselius electrophoretic technique. They also stated that the gamma-fraction was present in unfractionated casein in concentrations too low for unequivocal detection by this method.

A method based on filter paper electrophoresis was developed to determine quantitatively the components of the casein complex.

MATERIALS AND METHODS

Preparation of casein samples. Three samples of casein were prepared from a composite sample of milk (Macdonald College herd), according to the method of Warner *(15)*, except for two modifications: First, only one precipitation was carried out, to avoid possible losses of gamma-casein, and, secondly, alcohol and ether were not employed as drying agents. The casein was freeze-dried after thorough washings with water (from 2 to 5° C.). The three samples (Casein 1, Casein 2, and Casein 3), prepared in this manner, were analyzed for total nitrogen. The results obtained on a moisture-free basis were as follows: Casein 1—15.41%; Casein 2—15.30%, and Casein 3—15.39%.

Received for publication February 27, 1957.

Azocarmine B. This was obtained from National Aniline Division, Allied Chemical and Dye Corporation, New York. Preparation of the dye solution and staining were performed as described by Harders and Van Mulken *(6)*.

Naphthalene Black 12B 200. This was obtained from Canadian Industries Ltd., Chemical Division, Montreal, P.Q., Canada. The commercial dyestuff was purified by the method of Kawerau *(9)*. Preparation of the dye solution and staining were performed as described by Flynn and DeMayo *(4)*.

Electrophoretic method. The method was essentially that of Durrum *(3)*, as modified by Flynn and DeMayo *(4)*. Strips of Whatman 3 MM. paper (8 × 16 in.) were dipped in veronal buffer (pH 8.6, ionic strength 0.05), so that the paper was saturated with the buffer solution. The wet paper was then placed in the appropriate position on a pebbled Plexiglass support *(13)*, so that both ends of the paper sheet dipped into the buffer compartments to the same depth of buffer. Approximately 0.4 ml. of the casein solution (0.05 g. casein per ml. of veronal buffer) was applied at a distance of 4 in. from the cathode end. Potential was maintained at 200 volts, giving a current of from 0.5 to 0.7 ma. per inch-width of paper. Electrophoresis was continued for a period of 16 hr. The apparatus was kept in a constant-temperature room at from 5 to 7° C. At the end of the run, the paper was dried at room temperature, or at 110° C., for 30 min., and before staining was cut into strips approximately 1 in. in width. The same results were obtained with both drying methods.

Photometry. Stained strips were scanned on a Photovolt Densitometer Model 525, using a 495-mμ filter with Azocarmine B and no filter with Naphthalene Black. Some workers *(5, 10)* have assumed that the light absorption by dyed protein in filter paper obeys Beer's Law. The validity of this assumption was tested experimentally by the method described by Crook, Harris, Hassan, and Warren *(2)*, in connection with serum analysis. Calibration curves obtained with Azocarmine B and with Naphthalene Black are shown (Figures 1 and 2). Identical calibration curves were obtained by dipping the strips in serial dilutions of the dye solution. This finding agrees with that of Crook, Harris, Hassan, and Warren *(2)*. Figures 1 and 2 demonstrate that an appreciable deviation from Beer's Law occurs even at quite low dye concentrations. Correction tables were prepared for both dyes, for readings which fell outside the linear range of the curves, and these corrected readings were employed in the determinations of the areas of the peaks.

Several methods have been proposed to determine the dividing line between adjacent components. The oldest of these methods *(14)* involves simply drawing a perpendicular from the trough of the curves to the base line, and regarding the area enclosed between the adjacent pairs of perpendiculars as a measure of the amount of a particular fraction. This method was followed in the present work.

RESULTS

Determination of the electrophoretic components of casein by direct photometry. Eight electrophoretic runs were performed on each of the three casein

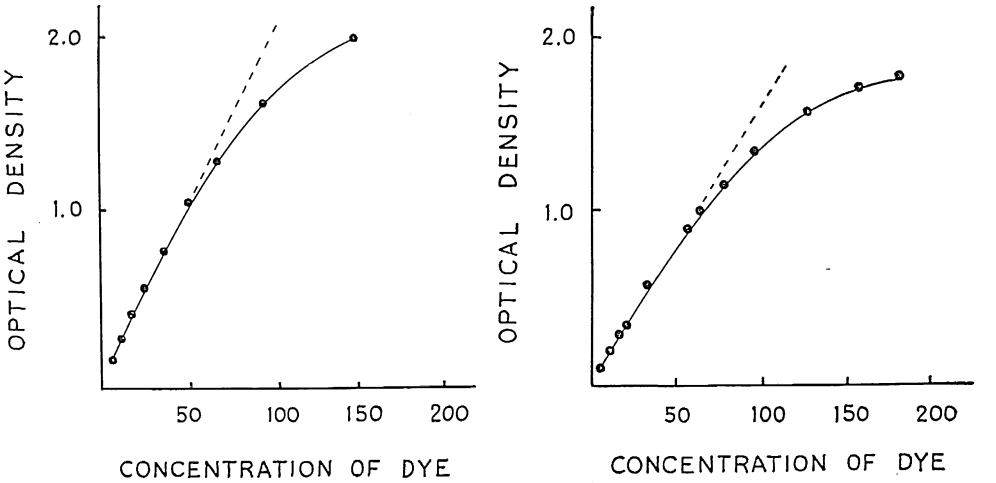


FIG. 1. Calibration curve for Azocarmine B. Broken line represents extrapolation of rectilinear portion of curve.

FIG. 2. Calibration curve for Naphthalene Black. Broken line represents extrapolation of rectilinear portion of curve.

samples (Casein 1, Casein 2, and Casein 3). The sheets were dried at room temperature. Three strips were cut from each sheet and the resulting 24 strips were stained with Azocarmine B. Eight similar runs were carried out for each of the casein samples and the 24 strips were stained with Naphthalene Black. The strips were scanned on the densitometer, and electrophoretic diagrams were prepared by plotting optical density against the distance along the strip. Corrections were made for readings which fell outside the linear range of the cali-

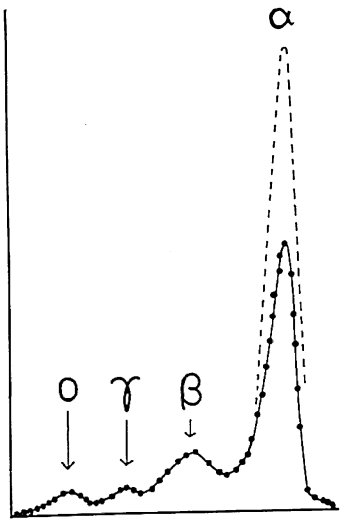


FIG. 3. A typical electrophoretic diagram of casein: The vertical axis represents the optical density and the horizontal axis represents the distance along the strip. The broken line represents values corrected for deviation from rectilinearity.

TABLE 1

Results for the determination of the electrophoretic components of casein*

Casein fraction	Azocarmine B			Naphthalene Black		
	Casein 1	Casein 2	Casein 3	Casein 1	Casein 2	Casein 3
	(%)	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Alpha	71.7 (2.2,[a] 3.1[b])	70.2 (2.0,[a] 2.8[b])	72.2 (1.9,[a] 2.6[b])	70.1 (1.7,[a] 2.4[b])	70.4 (1.3,[a] 1.8[b])	71.2 (2.9,[a] 4.1[b])
Beta	24.6 (2.9, 1.2)	24.5 (3.8, 15)	21.9 (1.7, 7.8)	23.9 (0.9, 3.8)	20.6 (1.7, 8.2)	20.5 (1.8, 8.8)
Gamma	2.3 (0.5, 22)	3.3 (1.4, 42)	3.4 (0.7, 21)	3.0 (0.9, 30)	5.3 (1.1, 8.2)	5.4 (1.6, 30)
Line of application	1.4 (0.4, 29)	2.0 (0.9, 45)	2.5 (0.7, 29)	2.4 (0.3, 12)	3.7 (0.5, 13)	2.9 (0.9, 31)

* Percentage results for each fraction represent the average of 24 determinations.

[a] Standard deviation.

[b] Coefficient of variation.

bration curves. A typical diagram is shown (Figure 3). The areas under each peak are reported as a percentage of the total area. The results of eight electrophoretic runs on each of the three casein samples, and for each of the two dyes, are presented (Table 1). The values for each fraction represent the average of 24 determinations (three strips cut from each of eight electropherograms).

Determination of the electrophoretic components of casein by nitrogen. The electrophoretic components of casein have been shown to contain approximately the same percentage of nitrogen *(7)*; hence, it is possible to estimate the casein components separated on paper by estimation of total nitrogen.

Three electrophoretic runs were carried out on each of the three casein samples. The casein solutions (5%) were applied to the paper so that each half of the 8-in. sheet contained 10 mg. of protein. After the run, the paper sheets were dried at room temperature and cut in half. One half was cut into strips and stained in the usual way, to determine the position of the separated components on the paper; the remaining half was used for the nitrogen determinations. Boundaries of the fractions, as defined by the mid-line of the trough between the peaks of the fractions, were marked by a pencil-line. The width of each band was measured, in order that an appropriate blank nitrogen value could be calculated. Excess buffer was removed from the paper by three ten-minute washings in methanol. These washings reduced the blank value to that given by untreated filter paper. The bands were cut out as whole sections and total nitrogen was estimated on each fraction by a micro-Kjeldahl method *(1)*. Two filter paper strips, 1 in. in width, were cut from each paper (8 × 16 in.) outside the protein bands, and were digested and the nitrogen determined in the usual way. An average blank per inch-width of paper was calculated from the results. Results of the nitrogen analyses on the samples of casein are given (Table 2). Each result represents the average of three determinations.

Correlation of the quantitative results obtained by direct photometry on stained paper with those obtained by total nitrogen analysis. The alpha-, beta-, and gamma-fractions of the casein complex were prepared from a sample of casein *(15)* by the methods of Warner *(15)* and of Hipp, Groves, Custer, and McMeekin *(8)*. A 1% solution of each of the casein fractions, prepared by dis-

TABLE 2

Results for nitrogen determination on the electrophoretic components of casein

Casein fraction	Casein 1 1.41 mg.N applied, 90% recovered		Casein 2 1.48 mg. N applied, 94% recovered		Casein 3 1.45 mg. N applied, 101.3% recovered	
	Nitrogen recovered	Recovered total nitrogen	Nitrogen recovered	Recovered total nitrogen	Nitrogen recovered	Recovered total nitrogen
	(mg.)	*(%)*	*(mg.)*	*(%)*	*(mg.)*	*(%)*
Alpha	0.79	61.7	0.80	56.4	0.85	57.8
Beta	0.41	32.0	0.48	33.8	0.45	30.6
Gamma	0.06	4.7	0.08	5.6	0.12	8.2
Line of application	0.02	1.6	0.06	4.2	0.05	3.4

solving the proteins in veronal buffer (pH 8.6, ionic strength 0.05), was applied in equal amounts to strips of filter paper, and electropherograms were prepared by the procedure outlined above. The papers were stained (Azocarmine B) and were scanned on the densitometer. Electrophoretic diagrams typical of those which were obtained are shown (Figure 4). The relative heights of the peaks

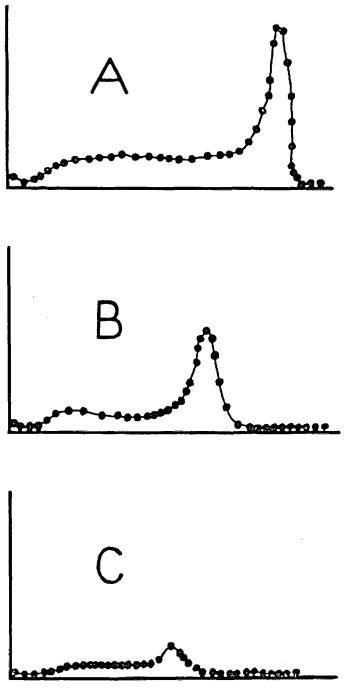


FIG. 4. Electrophoretic diagrams of casein components: A, alpha-casein; B, beta-casein, C, gamma-casein. The vertical axis represents the optical density and the horizontal axis represents the distance along the strip.

indicate that the casein fractions had different dye-binding capacities. The ratio of the amount of dye bound by the beta-component to that bound by the alpha-component, as determined by the area of the peaks, and calculated from average results from three electropherograms, was 0.63. Similarly, the ratio of the amount of dye bound by the beta-component to that bound by the gamma-component was 2.4. The whole experiment was repeated, using a 2% solution of the casein fraction, and identical factors were obtained. These factors (0.63 and 2.4) were used to correct the results given (Table 1) for the differences in dye-binding capacities of the three casein components. Calculations were based on the average of the results (Casein 1, Casein 2, and Casein 3) obtained with Azocarmine B. Results of these calculations are shown (Table 3).

DISCUSSION

From the foregoing results, it is evident that the alpha-, beta-, and gamma-fractions may be separated from casein by zone electrophoresis. The two dyes,

TABLE 3

Results corrected for differences in dye-binding capacities of the three casein components

Casein fraction	Per cent of total area[a]	Per cent of total mobile fraction[b]	Area corrected for difference in dye-binding	Corrected area expressed as per cent of total mobile fraction	Per cent of total mobile fraction based on Kjeldahl nitrogen
Alpha	71.4	72.8	45.9	59.3	60.5
Beta	23.7	24.1	24.1	31.1	33.2
Gamma	3.0	3.1	7.4	9.6	6.3
Line of application	1.9	—	—	—	—

[a] Average of results obtained with Azocarmine B (Table 1).
[b] The mobile fraction refers to the alpha-, beta-, and gamma-components.

Azocarmine B and Naphthalene Black 12B 200, enable the fractions to be detected qualitatively. Whenever electropherograms of the three different samples of casein were stained by the two dyes, it was found that the densitometric plots agreed as between the two dyes.

When the percentage distribution of the three fractions was measured on the basis of Kjeldahl nitrogen determinations, the values differed considerably from those based on the densitometric plots of staining, even when appropriate corrections were applied for departures from Beer's Law. However, when further corrections for the different dye-binding capacities of the different fractions were introduced, the corrected results for distribution based on staining agreed reasonably well with the distribution based on determination of Kjeldahl nitrogen. It is realized, of course, that replication of this agreement would call for checking of staining technique and densitometry against Kjeldahl nitrogen in each laboratory. We suspect, nevertheless, that the value of zone electrophoresis as a quantitative method for studying casein fractions may be considerable. Differences in the percentage distributions as between the three casein samples for staining density followed trends similar to those observed for Kjeldahl nitrogen. This is the more remarkable, in view of the relatively small range of variation for a given fraction.

Finally, the corrected densitometric distributions and the Kjeldahl nitrogen distributions now reported display a measure of agreement with the values recorded in the literature for distribution, based on the classical Tiselius method. Admittedly, the significance of correlations between the results of zone electrophoresis is still controversial. Nevertheless, the present results suggest that both methods isolate similar fractions of casein quantitatively as well as qualitatively.

SUMMARY

1. Alpha-, beta-, and gamma-components of the casein complex have been separated by electrophoresis on filter paper. Estimation of the separated protein fractions has been accomplished by direct photometry of the dyed proteins in filter paper.

2. Dye-binding capacities of alpha-, beta-, and gamma-casein, prepared by a chemical method, have been measured experimentally. With the staining pro-

cedure used, the components did not bind the dye in proportion to the amount of protein present. Correction factors based on the differences in dye-binding capacities of the protein fractions have been determined.

3. The photometric results, corrected for deviation from Beer's Law and for differences in dye-binding capacities, have been shown to agree with the results obtained by protein nitrogen analyses. The percentage composition of the casein components analyzed in this manner was of the following order: alpha-, 60%; beta-, 32%, and gamma-, 9%.

ACKNOWLEDGMENTS

The authors wish to thank the Agricultural Research Council, P.Q., Canada, for financial support which has defrayed part of the expense of this investigation.

The assistance of G. O. Henneberry and J. P. Julien (Dairy School, Ste. Hyacinthe, P.Q., Canada) in the preparation of the casein fractions is gratefully acknowledged.

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CLOTTING OF CASEIN WITH PEPSIN : AMOUNT AND NATURE OF THE SOLUBLE PRODUCTS

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SUMMARY

The amounts of soluble products resulting from the action of pepsin on whole casein at pH 6.5 have been determined by precipitating the solutions with $CaCl_2$ (clot formation), and with 12% trichloracetic acid, and by adjusting the pH to 4.7 with HCl. α- and β-Caseins were studied in the same manner; with the latter, however, no clot was obtained. In general, 2 to 5% of the whole casein and α-casein N became soluble by the action of pepsin, with the lower values obtained with trichloracetic acid as the precipitant. Parallel increases in soluble P were obtained. Both paper and column chromatography showed that the soluble fractions contained a number of components, none of which were free amino acids or orthophosphate. Increases in the soluble fraction of β-casein indicated that considerable proteolysis had occurred. Most of the soluble fractions did not pass through a cellophane membrane.

When rennin acts on casein under suitable conditions to give a clot with calcium ions, about 4% of the casein is no longer precipitated at the isoelectric point or with trichloracetic acid *(1)*. The nature of this soluble fraction is important for an understanding of the clotting reaction *(2)*. To initiate the present study of the clotting of casein, the same precipitating conditions (isoelectric, trichloracetic acid) have been studied, with pepsin as the clotting agent. [Rennin and pepsin are similar in their clotting action and NPN release, although this is not true for all proteolytic enzymes *(11)*]. The amount of nitrogen remaining in solution when the calcium clot is removed has also been determined. In addition, the amount of casein phosphorus remaining in solution with the three precipitating conditions has been measured. These methods have been applied to whole casein, and to α- and β-casein. The soluble fractions have also been subjected to dialysis and chromatography to determine their nature.

MATERIALS AND METHODS

Casein. The whole casein was precipitated from skimmilk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid *(6)*. The casein was finally dried with absolute ethanol and ether.

α-Casein and β-casein. These caseins were prepared from whole casein by the urea fractionation procedure *(7)*. All of the caseins were stored in a desiccator at a relative humidity that maintained the moisture content at 10.0%.

Received for publication August 27, 1957.

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The concentrations given are for moisture-free products. The solutions were prepared by dissolving the isoelectric caseins with the addition of sodium hydroxide.

Paracasein. The casein was clotted with pepsin, as described below. A suspension of the clot was adjusted to pH 4.7 and washed to reduce the calcium concentration. It was dissolved at about pH 7 and reprecipitated several times at pH 4.7. The paracasein was finally dried with absolute ethanol and ether.

Pepsin. The pepsin was a crystalline product obtained commercially (Armour).[2] It was dissolved in a small amount of water for use.

Analytical methods. The nitrogen was determined by the Nessler method after digestion with sulfuric acid. Phosphorus was estimated with the ammonium molybdate reagent after reduction with ferrous sulfate, on samples that had been digested with sulfuric acid and hydrogen peroxide.

Chromatography. For paper chromatography, the solvent systems most frequently used were *n*-butanol:water:acetic acid (4:1:5), benzene:water:propionic acid (25:25:62), *n*-butanol:acetic acid:water:pyridine (30:6:24:20), and water-saturated phenol. These were the most satisfactory. Numerous others were tried with little success. Both ascending and descending one-dimensional paper chromatography was used. For column chromatography the resin Amberlite IR-120(H+) was used. Successive fractions were eluted as described later.

Color tests. Peptides were spotted on the papers with the ninhydrin reagent. For inorganic phosphate, the ammonium molybdate reagent was used with reduction by stannous chloride *(10)*. For organic phosphate, the same test was used but the ammonium molybdate reagent contained perchloric acid to release inorganic phosphate *(3)*. In a few instances, spots were obtained with this reagent where there was no corresponding ninhydrin reaction, suggesting that cyclic phosphate-containing peptides might be present, or perhaps carbohydrate-phosphate compounds.

Concentration of solutions. The solutions were concentrated by a rapid, large-surface technique *(4)* at from 30 to 35° C.

Dialysis. The solutions were dialyzed for 72 hr. at 7° C. in a cellophane sac against four volumes of water. The water was changed every 24 hr.

Action of pepsin on casein; methods of precipitation. The formation of soluble products by the action of pepsin on casein was measured after precipitation of the casein in three different ways: *(a)* Precipitation of the isoelectric point by adjustment of the pH to 4.7 with 0.1 *N* HCl. *(b)* Precipitation by addition of concentrated trichloracetic acid (TCA) to a concentration of 12%. [These procedures have been described by Alais *et al. (1).*] *(c)* Precipitation by clot formation with calcium chloride present (15 m*M* per liter). Unfortunately, with this procedure the soluble portion before pepsin treatment can not be determined directly as a control. To provide an indirect control, the soluble fraction was adjusted to pH 4.7 to precipitate any paracasein, and also

[2] Mention of commercial products in this article does not constitute a recommendation over similar products.

the solubility of a purified paracasein under the clotting conditions was determined. With the concentration of whole casein used (2%), the calcium ion clots obtained with 15 m*M* of $CaCl_2$ per liter were flocculent. When the solutions also contained 5 m*M* of phosphate per liter, good clots were obtained, typical of those obtained with skimmilk. The gel-like clot given by phosphate, originally described by Hammarsten *(5)*, is also obtained with α-casein.

The pepsin was permitted to act on 2% whole casein at pH 6.5 and 30° C. In experiments with α- and β-casein, the concentrations were 1%. Pepsin was added to whole casein in a ratio of 1:2,000 and to α- and β-casein in a ratio of 1:1,000. Calcium ion, when present, was obtained by the addition of 15 m*M* of $CaCl_2$ per liter. The reactions were stopped at the end of 5 min., except in the time-course experiments, by the addition of TCA or by heating the mixture for 15 min. at 100° C. The mixtures were then cooled at 25° C. and the precipitations completed. The solutions were clarified by centrifuging, and subsequent determinations (N and P analysis, etc.) were performed on this soluble fraction. For chromatography, the solutions were concentrated by the method mentioned earlier.

RESULTS

In preliminary experiments, the time required for maximum formation of the specific soluble fraction by the action of pepsin on casein was found to be about 5 min. for the conditions employed. In confirmation of the findings of Alais *et al. (1)*, when 12% TCA was the precipitant, slightly over 2% of the casein N became soluble in 5 min. (as shown in Table 1). There was no significant increase when the time was extended to 30 min. Alais *et al.* have shown *(1)*, with the use of 2% TCA as the precipitant, that a nonspecific hydrolysis occurs, as well, which continues to increase beyond 5 min.

In Table 1 are shown also the amounts of soluble fraction obtained with precipitation at the isoelectric point of pH 4.7, and with $CaCl_2$ alone as the precipitating agent without changing the pH (6.5). For the latter, the solubility without pepsin can not be obtained directly, but adjustment of the soluble fraction to pH 4.7 shows that very little is precipitated which might be casein. An estimate of the inherent solubility of paracasein was made with purified paracasein precipitated under the same conditions ($CaCl_2$, pH 6.5) (Table 1). Apparently, only about one-quarter of the soluble fraction can be paracasein. After correcting for this amount, it is found that when $CaCl_2$ alone is the precipitant, about 5% of the total N is made soluble by the action of pepsin. With the other precipitating conditions, the soluble fraction arising from the action of pepsin is obtained by subtracting the no pepsin value from the pepsin value. With precipitation at pH 4.7, the soluble fraction is 2.5%; with 12% TCA, only 1.7%. In general, parallel amounts of soluble P are obtained. With α-casein the respective amounts of soluble N are 3.5, 6.0, and 1.7%. β-Casein gave no clot with pepsin, but considerable proteolysis was evident from the 20% soluble at pH 4.7, and 3.2% soluble in 12% TCA.

TABLE 1

Increase in the soluble fraction (N and P) of whole casein, and α- and β-casein after treatment with pepsin at pH 6.5, determined with three precipitating conditions[a]

Precipitating conditions	Casein preparation	Treatment	Soluble fraction (casein = 100), %		Dialyzable (soluble = 100), %	
			Nitrogen	Phosphorus	Nitrogen	Phosphorus
Precipitated 12% TCA, $CaCl_2$ present	Whole	Pepsin	2.2	4.1		
Precipitated 12% TCA, $CaCl_2$ present	Whole	No pepsin	0.54	0.98		
Precipitated 12% TCA, no $CaCl_2$	Whole	No pepsin	0.23	0.98		
Precipitated pH 4.7, no $CaCl_2$	Whole	Pepsin	3.2	3.1	25.9	38.3
Precipitated pH 4.7, no $CaCl_2$	Whole	No pepsin	0.72	1.55	17.2	17.9
Precipitated pH 4.7, $CaCl_2$ present	Whole	No pepsin	1.63	2.3	9.5	16.4
$CaCl_2$, centrifuged pH 6.5	Whole	Pepsin	6.5	8.0	8.0	13.0
Soluble above, not precipitated pH 4.7	Whole	Pepsin	5.8	5.7	5.4	11.6
$CaCl_2$, centrifuged pH 6.5	Paracasein	.....	1.7	2.1		
Precipitated 12% TCA, no $CaCl_2$	α-Casein	Pepsin	3.7	5.9		
Precipitated 12% TCA, no $CaCl_2$	α-Casein	No pepsin	2.0	4.5		
Precipitated pH 4.7, $CaCl_2$ present	α-Casein	Pepsin	3.8	5.6		
Precipitated pH 4.7, no $CaCl_2$	α-Casein	Pepsin	7.1	8.2		
Precipitated pH 4.7, no $CaCl_2$	α-Casein	No pepsin	0.8	4.1		
$CaCl_2$, centrifuged pH 6.5	α-Casein	Pepsin	3.8	5.6		
Soluble above, not precipitated pH 4.7	α-Casein	Pepsin	3.5	5.2	17.6	34.9
Precipitated 12% TCA, no $CaCl_2$	β-Casein	Pepsin	4.0	5.5		
Precipitated 12% TCA, no $CaCl_2$	β-Casein	No pepsin	0.8	1.57		
Precipitated pH 4.7, no $CaCl_2$	β-Casein	Pepsin	21.6	8.75		
Precipitated pH 4.7, no $CaCl_2$	β-Casein	No pepsin	1.28	1.46		
Precipitated pH 4.7, $CaCl_2$ present	β-Casein	Pepsin	11.6	12.7	27.8	11.9

[a] The amount of each fraction dialyzing through a cellophane membrane is also shown.

Several of the soluble fractions were subjected to dialysis. The results (Table 1) show that most of the soluble fraction remained within the dialysis sac. This was the case even when the dialysis was carried on for several days.

The soluble fraction from a calcium chloride clot was concentrated and subjected to paper chromatography. By testing with several solvent systems, the fraction was found to be quite complex, consisting of at least ten components. The relative amounts of these components were not determined. None of the components were free amino acids or free phosphoric acid, judged by R_F values and specific color tests. Further, the fraction exhibited poor chromatographic behavior; some of the material remained at the starting point and strong tailing occurred, suggesting that some of the components were of rather high molecular weight.

A similar soluble fraction (96 mg. of N, 6.4 mg. of P) was separated into acidic, basic, and neutral fractions by chromatography on a 1 by 20 in. column of Amberlite IR-120(H+). The acidic fraction (17% of initial N and P) was in the filtrate and this was increased by about 20% by elution with 0.01 *N* HCl, the neutral fraction (52% of N, 38% of P) was eluted with 1 *N* NH_4OH, and the basic fraction (6% of N, 2.5% of P) with 1 *N* NaOH. Additional N and P were obtained in a P-rich compound (1.2% of initial N, 20% of P) which crystallized in the resin. The acidic fraction was hydrolyzed with hydrochloric acid. Paper chromatography of the hydrolyzed acidic fraction showed that there were 15 or more amino acids present, providing additional evidence that these components were of relatively high molecular weight.

DISCUSSION

Study of the action of pepsin on casein under the conditions required to give a clot with calcium chloride shows that 1.7% became soluble in 12% TCA, and 2.5% no longer precipitated at pH 4.7. In the case of α-casein, the respective values were 1.7 and 6.3%. These values are of the same magnitude as values reported by Nitschmann and coworkers for the action of pepsin *(11)* and rennin *(1)* on whole casein and α-casein *(13)*. These workers also observed that the minimum amount of soluble fraction was obtained with 12% TCA. The present studies have, in addition, shown that the decrease in solubility on formation of paracasein is such that $CaCl_2$ precipitation at pH 6.5 is feasible for removing the paracasein. The portion remaining in solution for whole casein and α-casein is 5.0 and 3.5%, respectively, about the same as that remaining in solution at the isoelectric point, pH 4.7.

The release of P during the clotting of casein has not hitherto been studied systematically, but the present studies show that the solubilization of P parallels that of N. None of it is inorganic, judged by spot test. This parallel release of N and P is surprising, in view of the probably uneven distribution of phosphate-binding sites in the casein. The parallel in these values does not necessarily mean that a portion of the casein is completely hydrolyzed, but may be entirely coincidental. Since the present studies were completed, Nitschmann

et al. (14) have reported that clotting of casein leads to the appearance of an unusual protein-polysaccharide component in the soluble fraction. This component contains 11.4% N and 0.57% P, or a molar ratio of 44, very close to the value of 41 for whole casein.

Although, in general, soluble N and P values were in parallel, when TCA was the precipitant the apparent solubility of P exceeded that of N. Lability of phosphate ester bonds to TCA was considered a possibility. However, in experiments entailing more prolonged exposure to TCA there was no increase in the soluble P. The inclusion of calcium chloride in experiments comparing the different precipitating conditions (Table 1) led to variable results; in some instances the soluble fraction was increased, in others decreased. A study of the action of rennin on casein *(8)* indicated that the soluble fraction was 34 to 120% greater for 16 than for 12 mM of $CaCl_2$ per liter.

Study of the soluble fraction by chromatography showed that it consists of ten or more components. A high molecular weight for some of the fractions is suggested by retention within a cellophane dialysis sac, and also by the presence of many amino acids in these components, suggesting that a fairly large polypeptide must be represented. Polymerization of small peptides into larger peptides during manipulation (plastein formation) is a possibility, but the presence of the large number of amino acids indicates that if this did occur, the resulting polymer could not have arisen from a single peptide. Results as a whole suggest that the soluble fraction arises from the rapid hydrolysis by pepsin or rennin of a number of susceptible bonds, giving rise to a number of relatively large peptides. The specificity of pepsin *(15)* suggests that the split will occur adjacent to phenylalanine or tyrosine. Wissmann and Nitschmann have observed *(17)* that phenylalanine appears as a new terminal group during the action of rennin on α-casein.

Alais *et al. (1)* had previously concluded that the NPN fraction arising from the action of rennin consisted of more than one peptide. By varying the TCA concentration, a fractional precipitation is achieved (12% TCA—1.5 to 2% NPN; 2% TCA—4% NPN). By means of chromatography and electrophoresis on paper, the fraction soluble in 12% TCA appeared to be principally a single substance, whereas the 2% TCA soluble portion contained several components *(1)*. Keller *(9)* observed about eight peptides on chromatography of the fraction soluble at pH 4.7, obtained by the action of rennin on whole casein and α-casein. Much of this fraction, too, remained at the starting point.

The proteolysis of β-casein observed in the present study is considerably greater than the approximate 0.5% in a period of 5 min. observed by Nitschmann and Keller *(13)* with 12% TCA as the precipitant. In the same time, the specific proteolysis of α-casein attained 1.6% *(13)*. The casein preparations, however, show variability; in this same study, for example, Nitschmann and Keller had one sample of α-casein become soluble to the extent of 3%. This variability might account for the divergent results with β-casein. Because of the nonspecific hydrolysis of β-casein, there would appear to be advantages in using

α-casein rather than whole casein in studying the clotting reaction, for fewer components would be expected in the soluble fraction than in whole casein. Further, with minimum clotting time these components would represent principally those arising from the specific split leading to clotting.

The protein-polysaccharide compound reported *(14)* to be released by the clotting of casein is nondialyzable, with a molecular weight of from 6,000 to 8,000. It is strongly hydrophilic, containing not only 40% of polysaccharide but large amounts of serine and threonine. It is this hydrophilic property which is considered to bestow on natural casein its solubility in the presence of calcium ions.

The recent studies of Waugh and von Hippel *(16)* and McMeekin, Groves, and Hipp *(12)* leave no doubt that the α-electrophoretic component of casein is inhomogeneous. However, the amount of these new components [kappa-casein, for example, is reported *(16)* to be 15% of whole casein] makes it unlikely that they represent the protective colloid in natural casein which is split and solubilized during the clotting reaction. The recent report of Nitschmann *et al. (14)* appears to provide a more plausible explanation for the clotting of casein. The 1.5 to 2.0% of casein which becomes soluble during the clotting reaction is split from a major component of α-casein, and the unusual, strongly hydrophilic properties of the split fraction bestow on natural casein its solubility in the presence of calcium ions. The effect of rennin on kappa-casein *(16)* suggests that this fraction of α-casein may have the hydrophilic protein-polysaccharide bound to it.

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LABORATORY STUDY OF FACTORS INFLUENCING MILKSTONE FORMATION[1]

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SUMMARY

Through use of an Autotechnicon, an instrument which dips test pieces into and out of a series of solutions, a reproducible method for forming milkstone on stainless steel was developed. Milkstone quantity increased with increase of temperature of water used to rinse milk; increasing the drying time before or after this rinse did not influence milkstone formation. Interrelated chemical factors involved in producing milkstone were milk, water hardness, chlorine solutions, and cleansers. Water hardness was singly the most important factor and a nonlinear increase of milkstone with increasing water hardness was demonstrated. There was little difference in milkstone amounts formed either with sodium or with calcium hypochlorite; formation of less milkstone with chlorinated trisodium phosphate possibly could be attributed to water softening caused by hard-water salt precipitation. Of the cleansers tested, least milkstone was produced with an acid, slightly more with a neutral nonionic detergent, still more with one containing trisodium phosphate and pyrophosphate. Comparison of synthetic detergents indicated that a cationic helped produce about twice the milkstone formed with a nonionic or an anionic. Inorganic cleanser constituents producing clear hard-water solutions helped form less milkstone than those producing cloudy solutions.

The word milkstone has been used to describe certain deposits forming on dairy equipment being utilized in both production and processing of milk and milk products. Milkstone has been variously described as any contamination-defying, routine, daily cleansing methods *(9)*, a product resulting from interaction of heat-precipitated milk film, chemical constituents of the water supply, and alkaline cleansers *(6, 8)*, a product of heat-precipitated milk solids and various chemical and physical factors *(5)*, possibly as a thin, metallic-like, dense film resulting from interaction of heat, air, and milk *(3)*, and lastly, as a result of improper cleansing *(1, 2, 5-9)*. The prevention or reduction of milkstone formation possibly may be accomplished through use of properly formulated cleansers and efficient cleansing methods *(1, 4, 7)*.

Laboratory milk films have been prepared which elucidated the effect of several physical factors on film formation *(3)* and the interaction of milk films with alkaline cleanser ingredients *(6)*. The laboratory method described in this paper simulates not only typical cleansing procedures but includes treatment of stainless steel with water rinses and sanitizing compounds, so that the effects of these materials also could be studied and evaluated. Thus, by inclusion of all or most of the known factors, and by studying the relative importance of each factor, it was hoped that a practical approach to prevention of milkstone formation could be formulated. Based on the results obtained, several possibilities for prevention or reduction of milkstone formation seem feasible and are discussed later in this paper.

Received for publication September 3, 1957.

[1] This research was done for Pennsalt Chemicals Corp., at Whitemarsh Research Laboratories, and published with their permission.

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MATERIALS AND METHODS

The instrument used was the Autotechnicon (The Technicon Company, New York City), designed primarily for automatic staining of histological tissue sections. The Autotechnicon consists essentially of a number of metal arms rotating slowly in a clockwise direction while ascending and descending into various positions, coming to rest after descent to each position. Type 302 stainless steel strips with a No. 4 polish on one side, 3 in. by 1 in. in size, were cleansed first with an acid cleanser and then with an alkaline cleanser, thoroughly rinsed in distilled water, and dried. The strips were then dipped into carbon tetrachloride, heated at 100–105° C. for ½ hr., cooled in a desiccator for at least ½ hr., and weighed on an analytical balance. Four strips were suspended on hooks in pairs, from two opposing arms of the Autotechnicon, so that the lower 2 in. of the strips were consecutively dipped into beakers containing: *(a)* 200 p.p.m. available chlorine solutions at room temperature; *(b)* raw tank-mixed whole milk, initially at 37° C. and allowed to cool to room temperature during the test; *(c)* water at room temperature used as the first rinse; *(d)* cleanser solution at 49° C., at 0.25% concentration unless otherwise indicated, and *(e)* a second water-rinse at 49° C.

The exposure time of each strip to each liquid was 70 sec., and the transit time from liquid to liquid was 35 sec. This sanitizing-cleansing cycle was repeated 30 times, the milk being replaced with a fresh supply at 37° C. after 15 cycles. The milk was stirred mechanically during each cycle, about 5 min. before the strips were dipped into it, by means of a stainless steel stirrer attached to an arm of the Autotechnicon.

At the end of the exposure period, the strips were held at room temperature for 10 min. The strips were then treated by total immersion in ethanol, ethyl ether, ethanol, ethyl ether, and petroleum ether, consecutively, the immersion time in each solvent being 1 min. The strips were dried at 100–105° C. for ½ hr., cooled in a desiccator for at least ½ hr., and weighed, the milkstone quantity being determined by weight difference. All of the results shown are the average total amount of milkstone on four strips; at least two runs were made, to give an average figure. The strips were handled with clean, dry forceps throughout the procedure.

Rinses and solutions were composed of, or prepared in, water of 300 p.p.m. hardness calculated as calcium carbonate, unless otherwise indicated. The water was prepared with calcium chloride and magnesium sulfate with a Ca:Mg ratio of 3:1. Water hardness was determined by the Betz titration method (W. H. and L. D. Betz, Philadelphia, Pa.).

RESULTS

The method just described, for determining amounts of formed milkstone, is limited to study of one condition during a single test, and does not allow the use of parallel control determinations. For this reason, it was necessary to establish the limits of error of the method. Using the same samples of calcium

hypochlorite as sanitizer, and a commercial alkaline cleanser (Cleanser A, Table 3) throughout, nine single tests were done at intervals during a 2-hr. period. The results, expressed as total milligrams of milkstone on four strips, were: 39, 40, 42, 30, 38, 44, 33, 44, and 43. It may be seen that the results usually were reproducible within a few milligrams, but that occasionally a low figure *(30, 33)* would be obtained. It was evident that for comparative purposes two or more test trials were necessary, in order to obtain a reliable average figure; therefore, all the results reported below were derived on this basis.

Results of a study of the effect on milkstone formation of rinse temperatures and drying time, before or after the first rinse, are shown (Table 1).

TABLE 1

Relation of temperature of rinses and drying time, before and after first rinse, to milkstone formation[a]

Temperature		Time of drying		Av. milkstone
1st rinse	2nd rinse	Before 1st rinse	After 1st rinse	
		(sec.)		(mg.)
Room	49° C.	35	35	40
49° C.	Room	35	35	74
Room	49° C.	35	10	32
Room	49° C.	10	35	41

[a] The sanitizing-cleansing cycle included calcium hypochlorite and 0.25% of a commercial cleanser composed of trisodium phosphate, pyrophosphate, carbonate, and an anionic synthetic detergent.

The data indicate that increase of the temperature of the first rinse resulted in a marked increase in the amount of milkstone. Increasing the time of drying after the first rinse caused the formation of slightly less milkstone, but an increase in the drying time before the first rinse caused no change in milkstone formation.

To determine the effect on milkstone formation of each component of the sanitizing-cleansing cycle, one or two components were replaced with hard water. The results (Table 2) indicate that replacement of cleanser, calcium hypo-

TABLE 2

Sanitizing-cleansing cycle component effect on milkstone formation

Calcium hypochlorite	Hard water	Milk	0.25% cleanser[b]	Av. milkstone
				(mg.)
Yes	Yes	Yes	Yes	40
Yes	Yes	Yes	No[c]	20
No	Yes	Yes	Yes	17
Yes	Yes	No	Yes	21
No	Yes	Yes	No	5
Yes	None[a]	Yes	Yes	5

[a] "None" signifies that distilled water was used for the solutions and rinses, in place of hard water.

[b] The commercial cleanser used was a mixture of trisodium phosphate, pyrophosphate, carbonate, and an anionic synthetic detergent.

[c] "No" signifies that the component was replaced with hard water.

chlorite, or milk produced about one-half the milkstone formed with all the components present in the system. Elimination of both calcium hypochlorite and cleanser, or replacement of the hard water in the entire system with distilled water and keeping all other components, resulted in very little deposition of milkstone.

As water hardness was shown to cause the greatest change of any of the single components of the cycle, a further study of this factor was made. Using calcium hypochlorite and alkaline Cleanser A (Table 3), it was found that

TABLE 3

Milkstone formation with different commercial cleansers[a]

Cleanser	Cleanser composition	Av. milkstone
		(mg.)
A	Trisodium phosphate, pyrophosphate, carbonate, anionic detergent	35
B	Polyphosphate, carbonate, anionic detergent	22
C	Neutral nonionic detergent	14
D	Phosphoric acid, nonionic detergent	9

[a] The sanitizing-cleansing cycle included a commercial calcium hypochlorite compound and a 0.25% concentration of the listed cleansers.

at zero p.p.m. water hardness, 5 mg. of milkstone formed, at 150 p.p.m. hardness, 14 mg., and at 300 p.p.m. hardness, 40 mg. was obtained. It became evident that the amount of milkstone increased in a nonlinear fashion with increasing water hardness.

A study of the effect of different types of commercial chlorine compounds used with alkaline Cleanser A (Table 3), showed that slightly more milkstone resulted from the use of calcium-free sodium hypochlorite than from use of calcium hypochlorite; whereas, least milkstone was obtained through the use of chlorinated trisodium phosphate. Actual figures were 38 mg. with sodium hypochlorite, 35 mg. with calcium hypochlorite, and 30 mg. with chlorinated trisodium phosphate. It should be pointed out that the sodium hypochlorite solution was clear and that the calcium hypochlorite formed a cloudy solution; whereas, the chlorinated trisodium phosphate caused a voluminous precipitate to form in the hard water, with a resultant softening of the supernatant. The demonstrated marked effect of water hardness on milkstone formation may, at least partially, explain the low result obtained with chlorinated trisodium phosphate.

A comparison of the amounts of milkstone obtained through the use of various types of commercial cleansers was made (Table 3). This experiment indicated that the most milkstone resulted through the use of a cleanser containing trisodium phosphate and pyrophosphate (Cleanser A), less milkstone produced with a cleanser containing polyphosphate (Cleanser B), still less with a nonionic detergent cleanser containing no hard-water precipitating salts (Cleanser C), and that least milkstone was obtained with an acid cleanser (Cleanser D).

In order to elucidate the role of individual cleanser chemicals, different types of organic synthetic detergents were compared. Using the detergents at a concentration of 0.01% and calcium hypochlorite as the sanitizer, the average milligrams of milkstone produced with alkyldimethylbenzyl ammonium chloride, a cationic, was 33, with an alkylaryl sulfonate (anionic), 15, and with a nonionic alkylaryl polyether alcohol, 14 mg. Thus, it may be seen that the cationic detergent helped produce over twice the amount of milkstone that the anionic or nonionic detergents produced, the latter two being about equal.

Investigation of the role of possible inorganic constituents of cleansers indicated that sodium bicarbonate and sodium hexametaphosphate, at the concentrations used, caused no more milkstone than the control hard water (Table 4).

TABLE 4

Effect of inorganic components of alkaline cleansers on milkstone formation[a]

Inorganic component	Cleanser solution at 49° C.	Av. milkstone
		(mg.)
None (hard-water control)	Clear	22
Sodium bicarbonate	Clear	22
Sodium hexametaphosphate	Clear	22
Sodium tripolyphosphate	Cloudy	25
Sodium pyrophosphate	Cloudy	33
Sodium bicarbonate plus sodium hexametaphosphate	Clear	24
Sodium bicarbonate plus sodium tripolyphosphate	Cloudy	28
Sodium bicarbonate plus sodium pyrophosphate	Cloudy	28

[a] The sanitizing-cleansing cycle included calcium hypochlorite and the listed inorganic chemicals each used as cleansers at 0.075% concentration.

Sodium tripolyphosphate and sodium pyrophosphate, at concentrations less than necessary for complete softening of the hard water as indicated by the cloudy solutions, helped to produce more milkstone than the water control, the pyrophosphate being the worst offender. Combination of sodium bicarbonate and phosphates indicated that the least milkstone was produced with sodium hexametaphosphate; whereas, with sodium tripolyphosphate and sodium pyrophosphate more milkstone was formed. It may be significant that clear solutions of the cleanser ingredients in hard water, indicating complete softening or no precipitation of hard-water salts, produced approximately the same amount of milkstone as the water control, whereas cloudy solutions produced milkstone in marked excess over the control.

DISCUSSION

The present study clearly confirmed the common belief that the formation of milkstone is a complex process involving many interrelated factors. The delineation of the effects of individual chemical factors, such as cleansers and cleanser ingredients, chlorine sanitizers, water hardness, and milk itself, emphasized that each played its role, dependent to various extents on the other materials.

The most important single chemical factor involved in milkstone formation was shown to be water hardness. As water hardness is a well known, although not entirely understood, entity, its predominance in influencing milkstone formation suggested several rational approaches to the solution of the problem of preventing or reducing milkstone deposits. As little difference could be demonstrated between various types of chlorine compounds, modifications of these materials seemed to offer, at best, only a partial solution to the problem.

A more fruitful approach seemed to be one directed at the proper formulation of dairy cleansers. The use of polyphosphates in amounts sufficient to produce clear hard-water solutions, in place of trisodium phosphate, pyrophosphate, or possibly other hard-water precipitating salts, was particularly notable in reducing the amount of milkstone formed. It should be realized that a limit, both chemical and economical, can be reached in the amount of water hardness that may be softened by means of polyphosphates (or organic sequestering agents) incorporated in alkaline cleansers. It is possible that, in very hard water, properly formulated alkaline cleansers may be used in conjunction with a chemical or physical water-softening process. The use of the latter process, however, has limited application because of space and cost considerations. A more practical solution seems to be the utilization either of neutral detergent cleansers containing no hard-water precipitating salts or of acid cleansers, as cleansers of both types markedly reduced milkstone formation.

It was determined also that the temperature of rinse waters played a large role in milkstone formation. The results reported here parallel the common field experience that, if milk is rinsed from equipment surfaces with hot water, more milkstone forms than with a cold-water rinse. No evidence for a marked effect on milkstone formation by drying of milk films at room temperature was found.

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CHANGES IN TYPE CLASSIFICATION RATINGS OF IMMATURE HOLSTEIN FEMALES. I. THE OVER-ALL RATINGS[1]

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SUMMARY

Semiannual type ratings by official inspectors from 1945 to 1956 were analyzed to show some changes occurring in Holstein females of the New Jersey Agricultural Experiment Station from 6 mo. of age to the end of the first lactation. Ratings of 94 animals were grouped according to age, 6 to 18 mo. (yearlings), 18 mo. to freshening (2-yr.-olds), first lactation (first-calf heifers), and 6 mo. to freshening (nonadult) with each animal having two or more ratings in each period. Means of ratings decreased significantly with age from yearlings to 2-yr.-olds and from 2-yr.-olds to first-calf heifers. The correlation (0.16) between yearlings and first-calf heifers was not significant. Although significant, correlations between 2-yr. old and first-calf heifer ratings (0.41), and nonadult and first-calf heifer ratings (0.27), were also low. Random and confounded effects difficult to measure combined to account for from 45 to 54% of the total variation. Under the present rating systems, it does not seem practical to select for type at early ages.

Improved type in dairy cattle is of definite economic value and held in high esteem by the commercial and purebred breeder. Type ratings are accepted as being positively associated with milk production and even constitutional strength, and the more desirable type animals command higher prices in the sales ring. Working through official type classifiers, the purebred dairy cattle associations have fostered type and herd classification throughout their respective breeds.

Although under the present systems only adult animals can be officially classified, progressive breeders have been constantly searching for practical methods of selecting and culling females at an early age. This investigation was undertaken to explore the possibilities from scoring young Holstein females, by measuring the changes taking place in over-all type ratings, determining some of the causes of these changes, and evaluating the practicability of using nonadult ratings in predicting ratings of first-calf heifers.

By classifying heifers at 6-mo. intervals to about 24 mo. of age, Harvey *et al. (2)* obtained correlations between ratings of + 0.37 when different classifiers were used, and + 0.40 when the same classifier was used. Previously, Hyatt *et al. (3)* had estimated a correlation of +0.30 between any classification rating before and after calving; the correlation between combinations of ratings was +0.40.

When ratings made at less than 10 mo. of age were omitted, Johnson and Lush *(4)* obtained repeatability estimates of + 0.34 for official inspectors' ratings, and + 0.55 for station members' ratings. Consecutive ratings were only slightly,

Received for publication August 19, 1957.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Industry, New Brunswick. This study was part of a joint project between the Holstein-Friesian Association of America and the New Jersey Agricultural Experiment Station.

if at all, more alike than ratings separated by 2, 3, or 4 yr. Benson *et al. (1)* estimated a repeatability of $+0.56$ on single final type ratings of Ayrshire cows with repeat classifiers, and a repeatability of $+0.48$ with different classifiers. With heifers, corresponding estimates were $+0.28$ and $+0.20$.

Differences in average ratings of 19 Ayrshire cow facilities were found to be small by Tabler *et al. (8)*, but the variation among families was larger than that within families. Sire influences were highly significant and included both genetic and environmental variations. When these sire effects were removed, the among-family–within-sire variation was not significant.

By studying ratings made by different judges, McGilliard and Lush *(5)* reported average correlations of $+0.11$ for over-all ratings made annually on heifers prior to freshening, $+0.25$ when made at least once before and once after freshening, and $+0.60$ when made at least twice after freshening.

EXPERIMENTAL PROCEDURE

Data covering 11 yr. (1945 to 1956) were available for this study from official type classifications of the Holstein-Friesian herd owned by the New Jersey Agricultural Experiment Station. During this time, all females over 6 mo. of age were classified twice annually, generally in May and October, at the beginning of the pasture and winter feeding seasons, respectively. Since only females which have freshened at least once can be officially classified, all ratings of animals before freshening were unofficial, though made in a similar manner by the same classifiers.

Prior to the analyses, a system of grouping the data was devised to meet certain prescribed conditions. To smooth temporary fluctuations caused by season, pregnancy, lactation, and standards of inspectors, the data were grouped in age classes, with at least two ratings averaged for each animal in each class; the analyses being, therefore, analyses of average values of ratings during a given time period. These averages were considered to be the best estimates of the average type of each cow while it was in each age period. Three specific age groups were formed: Group A consisted of ratings made between 6 and 18 mo.; Group B, between 18 mo. and first freshening, and Group C, between first and second freshening. In the analyses, a fourth group, Group AB, was made up of all ratings from 6 mo. to freshening. To qualify, each animal must have been classified at least twice in each age group. Ninety-four females by 21 sires met all of the above requirements and were included in the investigation. The relatively high number of sires represented must be attributed to the presence of an artificial-breeding stud connected with the herd.

As often happens in breeding studies covering an extended period of time, a number of confounding factors became involved. In the early part of the study (1945 to 1950), classification ratings were made by three different inspectors, working simultaneously and independently. The size and significance of these inspector influences were described for this population by Wilcox *et al. (9)*. Subsequent to this period, only one inspector classified the herd at each classi-

fication. Every inspector returned at least once, and some more often than others. In a number of instances, an inspector returned while the animal was in the same age group; at other times the animal was in the next age group.

In the cases where three inspectors were involved, the mean of the three ratings was considered to be the actual rating of the individual. The effects of the inspectors repeating themselves one or more times have not been isolated for this study.

In the statistical analyses, standard analytical procedures were used. For ease of analysis and maintenance of equal intervals between the various scores, the classification ratings were coded as follows: Excellent rated as 6, Very Good as 5, Good Plus as 4, Good as 3, Fair as 2, and Poor as 1. A study of methods of estimating heritability of type classifications by Tabler and Touchberry *(7)* indicated this to be a sound procedure. With slight modifications, it was used successfully by McGilliard and Lush *(5)*.

RESULTS

Variance analysis. To estimate the size and significance of various effects upon changes in over-all type ratings, four specific analyses were undertaken. These involved within-animal comparisons. Group A *vs.* C (as yearlings and as first-calf heifers), B *vs.* C (as 2-yr.-olds and as first-calf heifers), AB *vs.* C. (all nonadult ratings and as first-calf heifers), and A *vs.* B (as yearlings and as 2-yr.-olds). Since the same 94 females were involved throughout the study, each group was comparable to the other three. The results of these analyses are summarized (Table 1). Shown in the table are the sources of variation of the average type ratings, with mean squares and components of variance in percentage.

TABLE 1

Mean squares and per cent of variance of over-all type ratings made at various ages

Source of variation	Degrees of freedom	Comparisons between groups: A *vs.* C. yearling and 1st-calf heifer		B *vs.* C. 2-yr.-old and 1st-calf heifer		AB *vs.* C. nonadult and 1st-calf heifer		A *vs.* B. yearling and 2-yr.-old	
		M.S.	*V.* %	*M.S.*	*V.* %	*M.S.*	*V.* %	*M.S.*	*V.* %
Age	1	5.68[a]	11.9	1.71[a]	4.0	4.74[a]	11.7	1.15[b]	2.8
Sire	20	1.48[a]	32.7	1.58[a]	43.1	1.47[a]	39.0	1.50[a]	42.6
Sire × age	20	0.30	1.6	0.23	1.1	0.27	4.4	0.22	1.5
Daughters within sires	73	0.07	2	0.04	0	0.05	0	0.15	0
Residual	73	0.27	53.9	0.21	51.8	0.19	44.9	0.19	53.2

[a] Significant at the 1% level.
[b] Significant at the 5% level.

Mean ratings of the four age groups were: Group A, 3.58; Group B, 3.42; Group C, 3.23, and Group AB, 3.49. Their standard deviations were 0.59, 0.68, 0.54, and 0.51, respectively, with comparable coefficients of variation of 16, 20, 17, and 15%. Bartlett's test of homogeneity *(6)* showed that the variability in Period B was greater than in Period A which, in turn, was greater than in C.

Age effects were highly significant, with the exception of the A *vs.* B comparison, where only a significant estimate resulted. As might logically be expected, the greater the range in ages, the greater the per cent of total variance attributable to age. Only 4.0 and 2.8% resulted in the Group B *vs.* C and Group A *vs.* B comparisons, respectively; whereas, 11.9 and 11.7% resulted from the Group A *vs.* C and Group AB *vs.* C. comparisons.

The among-sire source of variation reflected the differences among the sire-group means. These estimates of sire influence accounted for 32.7, 43.1, 39.0, and 42.6% of the total variation in each of the four comparisons, respectively. As each animal is represented in each age period, and each age period in three of the four comparisons, these are not independent estimates. Since the AB *vs.* C comparison includes all data, its value (39.0%) is the most reliable and lies, as expected, between the A *vs.* C and B *vs.* C values.

Though not shown in the table, an additional analysis of the four age groups individually showed that sire influence (the among-sire–within-group variation) was highly significant in each case.

There were no indications of a real sire-by-age interaction. With the groupings used here, daughters within sires varied less than might be expected, giving negative components which were called zero. The residual term represented primarily a combination of random error, inconsistent changes among animals between age periods, and differences among classifiers in rating an animal at different ages. The combined effects accounted for from 45 to 54% of the total variation.

Correlation analysis. The relationship and significance of the ratings made at the different ages were measured by standard correlation procedures. The same four comparisons were made as in the analysis of variance. The correlation of the type estimates of yearlings (Group A) and first-calf heifers (Group C) was + 0.16 (not significant). Significant correlations resulted with *(a)* A *vs.* B, + 0.40, *(b)* B *vs.* C, + 0.41, and *(c)* AB *vs.* C, + 0.27.

DISCUSSION

Definite effects on over-all rating can be attributed to age. A systematic influence did exist, with the trend downward as animals aged. Since a shorter time lapse occurred between A and B, and B and C, than between A and C, the greater age influence observed for the latter could logically be expected. This trend is quite rapidly demonstrated by the mean levels of ratings. It may be due to a better appearance of the animals at this age or to greater leniency on the part of the inspectors.

The differences in the amount of variation within Groups A, B, and C may be attributable to one, or both, of two factors. Either there is less actual variation in type of the first-calf heifers, i.e., they are more uniform than the young animals, or the inspectors tend to stay closer to the mean of the group because the ratings are official—as opposed to ratings of nonadult animals, which are unofficial.

The largest influence on over-all classification rating was associated with the groups of daughters of the different sires. In the case of the AB *vs.* C comparison, it amounted to 39%. Heritability calculated from values higher than 25% give impossibly high estimates. Thus, it is clear that some factors other than genetic have combined to make this value high, and refinement of the methods, such as consideration of time trends, characteristics of specific classifications, and differences in levels of ratings by individual inspectors, would be necessary to arrive at a valid estimate. This was not an objective of the study, but there is an indication that real sire differences probably do exist.

Apparently, there were no specific sire-age interactions of measurable size. At least among the sires studied, there was little tendency for daughters of one sire to improve with age while daughters of another became less desirable.

The small differences among daughters of the same sire are at least partially attributable to the existence of differences in the error term from one time period to another, and differences among inspectors between these time periods. The mean squares for among-daughters–within-sires include few of these effects, because the daughters tended to be contemporary, or nearly so, in most cases. While additional information might be desirable, further breakdown of the confounded effects making up the error term was impractical, in the light of the maintenance of adequate numbers.

Several points are apparent from a study of the correlation coefficients. The possibility of predicting accurately the over-all rating of the first-calf heifer from the ratings of the yearling is very small under practical conditions. This is shown by the fact that only 17% ($r^2 = 0.17$) of the variability in first-calf heifer ratings can be attributed to comparable variation in precalving ratings. Although a higher correlation would very probably result if all ratings were made by the same inspector, this would be difficult for a breeder to accomplish. Moreover, part of the increase in repeatability of the ratings might be caused by the unconscious recollection of previous ratings by the inspector. Greater success can be obtained by use of ratings made between 18 mo. and freshening. However, the animal is then of such an age that selection based on type classifications is difficult to carry out on a practical, economical basis. Although extremely poor-type animals are culled during this age period, these individuals are obvious, without being officially classified.

From a scientific standpoint, apparently it is true that type of first-calf heifers can be predicted more accurately from the ratings of the 2-yr.-old group than from all the nonadult ratings. At least, it can be said that there is no advantage for prediction purposes in including the ratings of the yearlings.

CONCLUSIONS

Type ratings of nonadult females are not sufficiently correlated with their ratings as first-calf heifers to justify selection for type at early ages. As the time interval between ratings increases, the correlation between ratings decreases. Mean ratings decreased with age and the differences in levels were statistically

significant. Differences among sire groups represented about 39% of the total variation, but this value undoubtedly contained other confounded effects.

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CHANGES IN TYPE CLASSIFICATION RATINGS OF IMMATURE HOLSTEIN FEMALES. II. THE SUBRATINGS[1]

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SUMMARY

Subratings of Holstein females of the New Jersey Agricultural Experiment Station from 6 mo. of age to the end of the first lactation, made by official inspectors semi-annually from 1945 to 1956, were analyzed. Ratings of 94 animals were grouped according to age, 6 to 18 mo. (yearlings), 18 mo. to freshening (2-yr-olds), first lactation (first-calf heifers), and 6 mo. to freshening (nonadults), with each animal being represented by two or more ratings in each period. Means of ratings of first-calf heifers were usually lower than ratings prior to freshening, although a systematic age influence often was not present. Differences in sire-group means were seldom significant; age-sire interactions were significant in some comparisons for some characteristics and in other comparisons for others. Ratings prior to 18 mo. are of little value in predicting ratings of first-calf heifers. Repeatability of average ratings between 2-yr.-olds and first-calf heifers ranged from 0.08 (dairy character) to 0.63 (rump). While more success may be obtained by using these later ratings, the relationships are still too low to justify selection for type in any but the most extreme cases.

Breeding for type in the dairy cow has gone further than the simple consideration of over-all rating. The dairyman frequently selects sires which are proven or believed to be prepotent for desirable mammary systems, large size, or strong and straight legs. The various breakdown components of the type score-card then come into prominence.

A limited amount of work has been published in regard to these individual characteristics. That some are more repeatable than others was brought out by Benson *et al. (1)*, who indicated a range of correlation coefficients from 0.30 to 0.61 for the different characteristics in a study of Ayrshire cows with repeat classifiers, and 0.23 to 0.48 for different classifiers. Corresponding ranges for heifers were 0 to 0.30 and 0 to 0.28. Because of low relationships between ratings of heifers prior to 18 mo. and after freshening, Porterfield *et al. (4)* concluded that selection for type at early ages would be impractical. As early as 1923, Gowen *(2)* noted that general appearance, size and character of udder, and size and shape of fore udder, rump, and barrel were the most difficult to evaluate. Definite differences were noted in the ease of rating of the various components by Wilcox *et al. (6)*. Mitchell *et al. (3)* have found positive relationships of varying degree between the subratings and both milk and fat production.

Received for publication August 19, 1957.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Industry, New Brunswick. This study was part of a joint project between the Holstein-Friesian Association of America and the New Jersey Agricultural Experiment Station.

This investigation was undertaken to determine the practicability of predicting type subratings of first-calf heifers from their ratings prior to freshening, by measuring the changes taking place and establishing several of the causes of these changes.

EXPERIMENTAL PROCEDURE

Analyses were carried out on the same animals included in a previous study *(7)*. Breakdown ratings of these 94 females were statistically treated in a similar manner. There the make-up of the several groups, the various influences isolated and not isolated, and the coding of the ratings were described. The analyses of variance and correlation techniques followed the same pattern as in the previous paper and as outlined by Snedecor *(5)*. Specific comparisons were made between *(a)* A and C, *(b)* B and C, *(c)* AB and C, and *(d)* A and B. The value for an animal in any age period represents the average of two or more ratings.

RESULTS

Variance analysis. Changes in ratings with age (Table 1) may be summarized as follows: General appearance declined slightly from A to B but there was no change from B to C; feet and legs, rump, and body capacity did not change; dairy character dropped markedly from A to B, with no change from B to C. Mammary system ratings as a whole did not change from A to B, but were lower after freshening (B to C). Fore udder declined significantly both from A to B and from B to C, whereas rear udder declined only after freshening.

TABLE 1

Means, standard deviations, and coefficients of variation of subratings grouped according to age

Rating	Group A			Group B			Group C			Group AB		
	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.
			(%)			*(%)*			*(%)*			*(%)*
General appearance	3.60	0.56	15	3.43	0.53	15	3.41	0.62	18	3.51	0.46	13
Feet and legs	2.92	0.73	25	2.80	0.61	22	2.92	0.70	24	2.86	0.53	19
Rump	3.46	0.64	19	3.43	0.69	20	3.55	0.73	21	3.44	0.62	18
Dairy character	4.23	0.41	10	3.89	0.46	12	3.91	0.50	13	4.04	0.33	8
Body capacity	3.89	0.56	14	3.83	0.56	15	3.91	0.63	16	3.85	0.44	11
Mammary system	3.55	0.57	16	3.47	0.58	17	3.27	0.59	18	3.51	0.45	13
Fore udder	3.62	0.47	13	3.49	0.42	12	3.29	0.66	20	3.55	0.36	10
Rear udder	3.51	0.63	18	3.56	0.57	16	3.31	0.64	20	3.54	0.48	14

The results of the tests of significance of these changes and the components of variance as percentages in the four comparisons are shown (Table 2). Where negative values were obtained for variance components, they are presented as zero. In terms of percentage of total variance, the largest change was in dairy character from A to B. Mammary system, particularly fore udder, also changed to a marked extent. Other subratings were little affected by age.

TABLE 2

Variance percentages and significance of all group comparisons for all classification subratings

Subrating	Source of variation				
	Age	Sire	Sire × age	Daughters within sires	Residual
	(%)				
Comparison 1. Group A *vs.* C					
General appearance	3.3	2.3	13.6[b]	15.1[b]	65.7
Feet and legs	0	2.5	14.5[b]	25.7[a]	57.3
Rump	0.2	10.2[b]	0	13.1	76.5
Dairy character	18.2[a]	1.2	6.2	14.7[b]	59.7
Body capacity	0	2.2	14.2[b]	22.3[b]	61.4
Mammary system	8.9[a]	0	10.0	18.3[b]	62.9
Fore udder	12.3[a]	0	15.1[b]	6.5	66.1
Rear udder	3.3	0	10.2	16.4[b]	70.1
Comparison 2. Group B *vs.* C					
General appearance	0	7.0	15.7[a]	31.7[a]	45.6
Feet and legs	0.9	0	5.9	50.6[a]	42.7
Rump	0.7	8.9	5.2[b]	54.2[a]	31.0
Dairy character	0	4.7	4.6	4.7	86.0
Body capacity	0.4	30.1[b]	0	20.3[a]	49.2
Mammary system	5.3[b]	0	6.0	36.9[a]	51.9
Fore udder	4.6[b]	0	11.9[b]	35.3[a]	48.1
Rear udder	6.4[b]	0	9.6[b]	41.6[a]	42.5
Comparison 3. Group AB *vs.* C					
General appearance	0.4	6.7	12.1[b]	25.4[b]	55.4
Feet and legs	0	6.9[a]	0	0	93.1
Rump	0.3	7.6	0	27.3[a]	64.8
Dairy character	3.3	7.2	15.4	8.0	76.0
Body capacity	0	8.9	6.5	27.6[a]	57.1
Mammary system	7.7[a]	0	7.5	29.8[a]	54.9
Fore udder	10.0[a]	0	9.7	19.5[b]	60.8
Rear udder	5.6[b]	0	8.8[b]	33.3[a]	52.3
Comparison 4. Group A *vs.* B					
General appearance	2.3[b]	2.2	0	0	95.5
Feet and legs	0.5	3.0	6.6	24.3[a]	65.6
Rump	0	9.1	0	34.0[a]	56.9
Dairy character	23.5[a]	3.4	0	12.0	60.9
Body capacity	0	0	6.4	51.9[a]	41.7
Mammary system	0.5	0	6.3	49.6[a]	43.6
Fore udder	3.7[b]	0	0.5	36.6[a]	59.1
Rear udder	0	0	9.4[b]	37.1[a]	53.5

[a] Significant at the 1% level.
[b] Significant at the 5% level.

With regard to three sources of variation, sire, sire-by-age interaction, and daughters-within-sire, comparison 3, AB *vs.* C, was considered most representative, because it contained all ratings for each animal.

Among sire-group means, the differences of feet and legs accounted for 6.9% of the total variation and were significant. Though not significant, general appearance, rump, dairy character, and body capacity showed approximately the same percentages, ranging from 6.7 to 8.9%. A test for the presence of different sire-age trends was made, and significant results were obtained in two

cases (general appearance and rear udder). The variation among daughters within sires was generally the largest of all the specific sources of variation, being positive in all but one case, and of appreciable size in most of the other cases.

Further breakdown of the residual term was not feasible in this study, although valuable information might well be contained in it. It doubtlessly contains variation among classifiers, variation created by blunt grouping techniques, and other less specific influences.

Correlation analysis. The relationships of ratings of the various ages were measured by standard inter-class correlation procedures. The same four comparisons were made and the resulting coefficients are presented (Table 3), with the results of the tests of significance. With one exception, all comparisons of

TABLE 3

Correlations between type ratings of various age groups for each subrating

Subrating	Group A *vs.* C	Group B *vs.* C	Group AB *vs.* C	Group A *vs.* B
General appearance	0.13	0.58[b]	0.29[b]	0.45[b]
Feet and legs	0.21[a]	0.50[b]	0.45[b]	0.26[b]
Rump	0.37[b]	0.63[b]	0.53[b]	0.43[b]
Dairy character	0.16	0.08	0.16	0.19
Body capacity	0.19	0.39[b]	0.37[b]	0.29[b]
Mammary system	0.10	0.28[b]	0.25[b]	0.39[b]
Fore udder	−0.01	0.32[b]	0.19	0.32[b]
Rear udder	0.08	0.40[b]	0.30[b]	0.32[b]

[a] Significant at the 5% level.
[b] Significant at the 1% level.

the youngest (A) and oldest (C) groups for each of the subratings resulted in positive coefficients. Yet, these coefficients were not significant except in the case of feet and legs ($P < 0.05$) and rump ($P < 0.01$). When the interval between the age groups was short (as with A *vs.* B or B *vs.* C), coefficients were significant in every subrating except dairy character.

As would be expected, all coefficients for the AB *vs.* C comparison lay at some point between the A-C and B-C comparisons. These coefficients were significant in every case except dairy character and fore udder.

DISCUSSION

A number of specific points can be brought out from these analyses of the individual subratings. The age influence follows a logical and well-defined pattern. Dairy character, which is difficult to describe and evaluate, changed most between the yearling and the 2-yr.-old. Systematic change in dairy character due to age was not evident between ratings made before and after freshening. Although this trend might be challenged, it most definitely was true in this study, since none of the total variation was attributable to this source. Udder changes, as described by mammary system with fore and rear udder, were evident in the mean values of the individual groups where little change occurred in the nonadult ratings, but a definite drop occurred with the first-calf heifers during the first lactation. With all other subratings, only general appearance in

the A *vs.* B comparison was significant, and here it accounted for only 2.3% of the total variation.

Ratings of dairy character were the highest of all subratings in each age group; yet, its standard deviation and coefficient of variation were the smallest. It is conceivable that, if the inspectors would use a wider range of the scale available to them, rather than staying close to a mean value, those ratings would be more highly repeatable. At least this appears to be true with feet and legs, where the range was greater and the correlations were higher. As expected, the means and standard deviations of the subratings fluctuated around the means and standard deviations of the over-all rating *(7)*. As with over-all rating, means of subratings of first-calf heifers tend to be lower than those of younger ages.

From the analysis of variance tables, it can be seen that the sire influence was generally small. These results are opposite to the results of the analysis of the over-all ratings, where significant effects were present in each comparison. The sires in this population apparently did not influence the subratings to any important degree. However, the sum total of these influences must have been great enough to show up in the over-all rating which, after all, is a weighted average of the subratings. The fact that individual sires do not significantly influence mammary system, body capacity, or dairy character will not be entirely accepted by either the research specialist or the purebred breeder.

The sire-age interaction term was significant in a number of cases. Here again, this is the direct opposite of the over-all rating. With the yearling and 2-yr.-old comparison, only rear udder was significant. This has doubtful practical application. On the other hand, general appearance interactions accounted for between 12.1 and 15.7% of the total variation in the three other comparisons and were significant in each case. Thus, different trends do exist among sires between age periods. These trends vary among the different subratings and are opposite to the trends existing with over-all ratings.

The influence of season on the interaction term is not clear. Means of spring classifications are usually slightly higher than those of fall in this herd. Studies are now in progress to measure this factor.

With few exceptions, the variation among daughters within sires was the largest specific source of variation. This large variation overshadowed the sire influence, which then failed to be significant or make up an appreciable percentage of the total variation.

The correlation analyses indicated that some of the characteristics are more fixed than others in the animals. At least, they are fixed in the sense that an inspector would rate the characteristic the same as a previous inspector. This among-inspector variation, in conjunction with inconsistent changes in type due to the time interval between the age groups, must be the important factor which makes these correlations small. The fact that a number of the correlations were highly significant does not belie the fact that only limited accuracy can be expected in predicting future ratings.

That some characteristics are more difficult than others to evaluate accurately has been shown in this herd *(6)*. Lower correlations could be expected with characteristics which are difficult to evaluate and/or which change irregularly with age. These two factors do not necessarily coincide. As a specific example, feet and legs were evaluated with more difficulty than most of the other subratings in this herd; yet, a higher correlation existed between their scores made at different ages than most of the other subratings.

As they stand, however, the correlations describe conditions existing in a practical type classification program that could be duplicated in commercial purebred herds. On this basis several conclusions can be drawn. With the exception of rump, and to a lesser degree, feet and legs, the type rating of a first-calf heifer can not be predicted with any reliability from her ratings between 6 and 8 mo. Dairy character can not be predicted between any two age periods covered in this study. As the time lapse decreases, prediction accuracy increases, with greater accuracy occurring between 24 mo. and first lactation than between 12 and 24 mo. A minor exception seems to be mammary system, in which the reverse is true, although fore and rear udders follow the rule.

CONCLUSIONS

It seems impractical to select animals for type as first-calf heifers on the basis of type subratings at early ages. Often of statistical significance, especially when the time interval is short, correlations between average type ratings of different age periods do not indicate a high degree of success in predicting future ratings. Few of the changes in type are associated with age alone, and sources of variation which are particularly difficult to measure make up a large part of the total variation.

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ESTRUS AFTER CONCEPTION IN A HERD OF HOLSTEIN-FRIESIAN CATTLE[1]

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SUMMARY

Estrus after conception was observed during 5.6% of 6,751 reproductive periods which terminated in nonabortive calvings. Cows in their second and third reproductive periods averaged 7.5%, as compared with little more than 4%, for animals in their first or more than seventh ($P < 0.05$). Of 1,905 cows calving one or more times, 18.3% showed estrus after conception at some period of their reproductive life. Estrus occurred after conception more than one time in 18% of the reproductive periods showing estrus after conception. Average length of estrous cycles after conception was 43 ± 1.9 days. The modal length of 21 days was observed in 5.6% of the 534 cycles observed. Fifty-five per cent showed estrus within 35 days after conception. Reproductive performance of cows during periods of postconception estrus was comparable to herd average, with respect to services per conception, calving interval, abortion, and retained placentae. Twinning was 1.2% higher than herd average. Percentage male calves (56.8%) was significantly higher than the average for the herd. Cystic tendency was observed at some time during the reproductive life of 70.5% of the cows observed having postconception estrus. The expected rate was 42% if the two phenomena were unrelated. Postconception estrus followed observance of cystic tendency in the same reproductive period 31.4% of the time, as compared with an expected rate of 17.6%. Posteonception estrus occurred most frequently in November, December, January, and June, and least frequently in March, July, August, and October. The monthly differences were not significant.

Occurrence of heat during pregnancy has been recognized in several species including the cow *(1, 3, 5, 6)* and sheep *(9)*. This phenomenon is of practical importance because of the likelihood, in the absence of accurate pregnancy diagnosis, that such cows are frequently sold as sterile. Such cows are also one cause of questionable paternity when a different sire is used for breeding at estrus after conception.

Branton *(1)* and Donald *(3)* reported that 3.4% of dairy cows exhibit estrus one or more times during pregnancy; whereas, Donoho and Rickard *(4)* observed a 6% rate. Donald *(3)* found that estrus occurred 15 to 232 days after conception and that sterility rates among those cows were below the herd average. Mirskaja *(6)*, using a vaginal-smear technique, observed that 21.7% of the cows showed indication of estrus after conception. Williams *et al. (9)* sacrificed 50 ewes within 90 days after conception. Eleven of these, or 22%, had shown estrus one or more times after conception. None of the ewes sacrificed shortly after a postconception estrus showed evidence of ovulation.

Received for publication June 18, 1957.

[1] Scientific Paper No. 1619, Washington Agricultural Experiment Stations, Pullman. Project W-2 (888). This research was financed in part under Regional Research, Research and Marketing Act, Section 9b3, Public Law 733.

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This study was undertaken to determine the incidence of estrus after conception as related to age, season, and other reproductive phenomena.

SOURCE OF DATA

Breeding records, including clinical records maintained by resident veterinarians for the Carnation Milk Farm herd of Holsteins from 1920–50, and under the supervision of one man since 1925, were used for this study. General information about the data has been published elsewhere *(7)*. Statistical procedures were according to Snedecor *(8)*.

RESULTS AND DISCUSSION

Postconception estrus was observed during 424 of 8,409 reproductive periods. As shown (Table 1), 378 (5.6%) of these occurred during 6,751 reproductive

TABLE 1

Percentage of cows and reproductive periods with estrus after conception

Cow group	Total	Estrus after conception	
Reproductive period basis	(No.)		(%)
Nonabortive calvings	6,751	378	5.6
Abortive calvings	636	29	4.4
Pregnant cows and heifers not observed until subsequent calving[a]	1,022	17	1.7
Total	8,409	424	5.0
Per cow basis			
Cows calving one or more times	1,905	348	18.3
Pregnant heifers not observed until subsequent calving[a]	624	5	0.8
Total	2,529[b]	353	14.0

[a] Determined as the result of positive pregnancy examination.
[b] Seventy-eight cows neither calved nor conceived during this study.

periods terminating in nonabortive calvings. Among the 424 reproductive periods 76, or 17.9%, involved postconception estrus more than once during the same reproductive period. Nineteen of the 76 (25.0%) showed more than two, ten cows had four, and one cow had eight. The total was 534 cycles. The tendency for pregnant cows to show more than one estrus after an initial estrus was approximately three times greater than the 5.6% expected (average for all reproductive periods terminating in nonabortive calving), if occurrence of a second estrus was random.

On a per-cow basis (Table 1) 348, or 18.3%, of those calving one or more times showed estrus after conception. Sixty-one of these 348 cows, or 17.5%, showed postconception during more than one reproductive period. Of these 61 cows, eight had postconception estrus in three, and one in four, different reproductive periods. There were 339 cows which had 1,320 reproductive periods (abortions included) following initial observance of estrus after conception. Among these reproductive periods 70, or 5.3%, again showed estrus after conception.

Although the tendency to repeat estrus within the same reproductive period exceeds expectancy, there is little evidence that cows showing postconception estrus in one reproductive period are more likely to repeat during later reproductive periods.

Distribution by reproductive periods. Based on the 5% confidence limits (Table 2), first reproductive period cows have a significantly lower rate of

TABLE 2

Incidence of postconception estrus by consecutive reproductive periods

Reproductive period	Total[a] calving	Postconception estrus total		$t_n 5\% \sqrt{\frac{p(1-p)}{n}}$
	(*No.*)[a]		(%)	
1	1,435	63	4.4	1.1
2	1,023	81	7.9	1.6
3	845	59	7.0	1.7
4	675	41	6.1	1.8
5	524	35	6.7	1.8
6	398	23	5.8	2.4
7	304	14	4.6	2.4
8	219	7	3.2	2.3
9	147	5	3.4	2.9
10	92	3	3.3	3.7
10	90	2	2.2	3.1
Total	5,752	333	5.8	0.6
Other cows[b]	999	45	4.5	1.3
All cows	6,751	378	5.6	0.5

[a] Abortions excluded.
[b] Purchased cows, and cows in herd prior to start of study.

estrus after conception as compared with second and third reproductive period cows. The average rate of estrus after conception was 3.6% for 852 cows with more than six reproductive periods. The 5% confidence limit was ± 0.6%. Cows of this latter age group were significantly lower in average rate of estrus as compared with cows in their second through fifth reproductive periods.

Interval distribution. Estrous intervals after conception show fewer than expected occurring in 18–25 days, as compared with repeat service cycle intervals for all normal cows in the herd (Table 3). Short cycles (2–17 days) were proportionately three times more frequent, normal cycles (18–25 days) one-half as frequent, and cycles longer than 100 days twice as frequent. The modal length was 21 days with 30, or 5.6%, occurring at this interval. This compares with 9.5% for the 5,363 repeat service cycles. The average length for the 534 cycles after conception was 43.3 ± 1.6 days, which is much less than the 78 days reported by Donoho and Rickard *(4)*.

Time since conception. The first postconception estrus occurred within 35 days after conception in 55% of the reproductive periods. Donald *(3)* observed 41% within 30 days and 54% within 45 days.

Cows which showed more than one estrus after conception within the same reproductive period had their first estrus within 35 days after conception in 55% of the cases. The interval from conception to first estrus in one reproduc-

TABLE 3

Postconception estrous intervals by normal and abnormal reproductive periods

Estrous interval	Normal[a]	Abnormal[b]	Total	Repeat service cycles[c]
(*days*)		(%)		
2–17	12.9	15.3	13.7	4.6
18–25	28.0	30.7	28.8	47.0
26–35	12.4	16.6	13.7	10.8
36–45	12.7	8.6	11.4	13.9
46–65	12.1	15.3	13.1	11.6
66–100	12.4	8.6	11.2	7.7
101–150	6.2	3.1	5.2	3.0
151–200	2.4	1.8	2.2	0.7
>200	0.8	0.0	0.6	0.5
Total cycles (*No.*)	371	163	534	5363

[a] Normal calving and no pathology observed during the reproductive period; estrous cycle length not considered.

[b] Abnormal calving or some pathology observed during the reproductive period.

[c] Normal reproductive periods for all cows in herd.

tive period ($\bar{x} = 48$ days) was not significantly correlated ($r = 0.18$; 59 *d.f.*) with a similar interval in another reproductive period ($\bar{x} = 53$ days). The correlation was also low ($r = 0.21$; 74 *d.f.*) between the first and second interval for 76 cows showing estrus at least twice after conception. The average was 42 days from conception to the first estrus and 41 days to the second.

The sizeable percentage of cows showing their first estrus after conception (42.5%) within 25 days presents a practical problem. If the cows were rebred to a different bull, it would be difficult to determine parentage. On a reproductive period basis, one could expect about three out of every 100 normal births to fall in this category.

Reproductive performance. In Table 4 cows showing postconception estrus are compared with herd average criteria of reproductive performance. In general, these cows conceived with slightly fewer services, had a comparable calving interval, and showed the same percentage of impaired fertility at disposal.

Calvings following postconception estrus were similar to herd average for percentage of normal single births. Twin births were higher than expected following postconception estrus, when one considers that the average age of calving was little higher than herd average (Table 4). Since cows showing postconception estrus also generally showed cystic tendency, a more detailed analysis of this relationship was made. Cystic tendency was rated into four groups as follows: A) cows with cystic follicles observed more than once, or having three or more short estrous cycles, each recurring in from two to 15 days in one or more reproductive periods; B) cows with cystic follicles observed once, or having three or more short cycles in one reproductive period, or two short cycles in more than one reproductive period; C) cows with two short cycles in only one reproductive period; and D) cows with less than two short

TABLE 4

Reproductive performance of cows during reproductive periods when postconception estrus occurred, compared with averages for the herd

Comparison	Reproductive periods with postconception estrus		Herd
	(No.)	*(av.)*	
Services per conception	424	2.4	2.5
Days from calving to first estrus[a]	283	62	64
Days in calving interval[b]	399	433	442
Gestation-terminating reproductive periods (days)	407[c]	271	272
Impaired fertility at disposal *(%)*	92[d]	34	34
Single births—normal *(%)*	325	78	78
Singles dead at birth *(%)*	10	2.4	3.7
Twin births *(%)*	22	5.4	4.2
Retained placentae *(%)*	50	12	10
Abortions *(%)*	29	7	9
Age at calving following postconception estrus (years)	407	6.4	6.1

[a] Data prior to 1926 excluded.
[b] Sixty days were subtracted from calving intervals of cows on advanced registry test, because these animals were bred for 365-day records and averaged 59 days longer to first service. Previous calving date was not always known for purchased cows.
[c] Seventeen cows were sold before calving.
[d] Does not include cows still in herd or those sold for dairy purposes.

cycles during their reproductive life in the herd. Under this system of grouping, each individual cow was assigned to a cystic tendency group. One cystic tendency group involving cows and the other involving reproductive periods were used to show this. Comparisons were made with the respective herd totals. There were 353 cows with postconception estrus and 249 of these (70.5%) showed cystic tendency during the same or during different reproductive periods while in the herd. Based on herd totals, 42.0% was expected. Some of the 353 cows had estrus after conception in more than one reproductive period. Therefore, there were 424 reproductive periods during which postconception estrus was observed. Cystic tendency preceded conception in 133, or 31.4%, of these reproductive periods. Based on herd totals, 17.6% was expected. More than one-half of these 133 cases was found in cystic tendency group (A) which, under the method used for cystic tendency classification, included the most obvious clinical cases. Little bias exists in this comparison. These data suggest a common physiological cause which must be considered when comparing sex ratios, twinning rate, and heritability.

Clapp *(2)* has previously shown that twinning rate is higher during reproductive periods involving cystic ovaries. It was shown (Table 4) that twinning rate was 5.4% for calvings terminating reproductive periods during which postconception estrus was observed. These same cows (Table 6) had a twinning rate of 4.5% for all other reproductive periods. Calvings terminating reproductive periods with postconception estrus showed a higher average twinning rate when in combination with cystic tendency. As shown in Table 6, Groups A and B averaged 7.1 and 6.7% twin births, respectively. The higher incidence of twinning is more related to multiple short estrous cycles and cystic follicles

TABLE 5

Relation of postconception estrus and cystic tendency

Variable	Total	Cystic tendency groups A		B		C		D		Total in cystic tendency groups A, B, and C	
	(No.)	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
					Herd totals						
All cows	2,607	465	17.8	411	15.8	218	8.4	1,510	58.0	1,094	42.0
All reproductive periods	9,994	975	9.8	532	5.3	248	2.5	8,203	82.4	1,755	17.6
					Postconception estrus study totals						
Cows[a]	353	125	35.4	82	23.2	42	11.9	104	29.5	249	70.5
Cystic in same reproductive period	424[b]	80	18.9	31	7.3	22	5.2	.....	.....	133	31.4

[a] Cows having postconception estrus and also cystic tendency during the same or during different reproductive periods.

[b] Some cows showed postconception estrus during more than one reproductive period.

(Groups A and B) than to whether estrus occurs after conception (see rates for Groups C and D). The 22 twin births produced by cows following postconception estrus showed unusual distribution, being composed of ten male, seven mixed, and five female sets. Other twin births by these cows were more normally distributed, being composed of 21 male, 31 mixed, and 14 female sets. The sex of six sets was unknown.

The percentage of males born after postconception estrus was 56.5%, as compared with 51.6% for the herd and 51.6% for other calvings by these cows. Using chi-square, this much deviation from the population mean could be expected approximately 5% of the time ($X^2 = 3.84$), when using population average of 51.6% males. Abortions and retained placentae did not vary from

TABLE 6

Influence of postconception estrus and cystic tendency on sex ratios and twinning rates

Cystic tendency	Cows showing postconception estrus: Calvings following: N for twins[a]	Twin births		N for sex[b]	Males		All other calvings: N for twins[a]	Twin births		N for sex[b]	Males	
	(No.)	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*
Group A	141	10	7.1	144	78	54.2	638	31	4.9	624	325	52.1
Group B	105	7	6.7	102	61	59.8	455	19	4.2	432	222	51.4
Group C	48	0	0.0	42	22	52.4	171	13	7.6	153	73	47.7
Group D	117	5	4.3	103	60	58.3	342	9	2.6	309	163	52.8
Total	411	22	5.4	391	221	56.5	1,606	72	4.5	1,518	783	51.6
Herd Total							7,376	311	4.2	6,720	3,471	51.6

[a] Includes abortions and other single births where sex was unknown.

[b] Male and female totals, including twins of known sex.

the herd average for cows showing estrus after conception. Thus, the reproductive mechanism shows little sign of impairment. The two most prominent features are differences in sex ratio and the likelihood of postconception estrus and cystic tendency occurring in the same reproductive period.

Monthly variation. The study of monthly variation in rate of postconception estrus involves several obvious sources for bias, namely: *a)* days pregnant; *b)* average number of cows in each pregnancy interval each month, and *c)* variable numbers of days in the months.

The likelihood of a cow showing estrus if pregnant only 2–15 days is considerably less than if pregnant 16–20 or more days. The 534 estrous cycles were distributed according to time the cows were pregnant when estrus was observed (Table 7). Dividing the percentage of cycles occurring within each

TABLE 7

Monthly variation in incidence of estrus after conception

Month	Pregnant cows adjusted[a]	Estrus after conception: Total	Estrus after conception: Av. per day	Estrus after conception: Pregnant cows/day	Distribution of estrus after conception: Pregnancy to estrous interval	Cases		Av. per day in interval	Adjustment[b]
	(No.)			*(%)*	*(No.)*	*(No.)*	*(%)*	*(%)*	*(factor)*
January	540	46	1.48	0.28 ± .08[d]	2–15	48	9.0	0.64	0.17
February[c]	550	42	1.49	0.27 ± .08	16–20	41	7.7	1.54	0.41
March	591	34	1.10	0.19 ± .07	21–25	101	18.9	3.78	1.00
April	595	46	1.53	0.26 ± .07	26–35	62	11.6	1.16	0.31
May	622	52	1.68	0.27 ± .07	36–45	55	10.3	1.03	0.27
June	663	56	1.87	0.28 ± .07	46–65	79	14.8	0.74	0.20
July	636	43	1.39	0.22 ± .06	66–100	72	13.5	0.39	0.10
August	622	39	1.26	0.20 ± .06	101–150	43	8.1	0.16	0.04
September	588	46	1.53	0.26 ± .08	151–200	22	4.1	0.08	0.02
October	551	36	1.16	0.21 ± .07	201–250	11	2.1	0.04	0.01
November	534	44	1.47	0.28 ± .08	Total	534			
December	539	50	1.61	0.30 ± .08					
Total	7031	534	1.46	0.25 ± .02					

[a] Adjusted to average equivalent number of 21- to 25-day pregnancies at midpoint of each month, using factors shown in last column of table.

[b] 21-25 day interval = 1.00.

[c] Days in month = 28.25.

[d] $t_n\ 5\% \sqrt{\frac{P(1-P)}{n \times \text{days in month.}}}$

interval by the number of days in each interval yields an approximate average rate for each interval as compared with other intervals. As shown in Table 7, cows pregnant 21–25 days were 5.9 times more likely to show postconception estrus than cows pregnant 2–15 days (3.78 ÷ 0.64). Since 21–25 days of pregnancy showed the greatest rate, it was assigned a factor of 1.00. It was then possible to derive factors to adjust cows in varying stages of pregnancy to a common basis. For example, the reciprocal of 3.78 ÷ 0.64 is 0.17, the factor necessary to adjust cows pregnant 2–15 days to a 21- to 25-day basis (Table 7).

The average number of cows pregnant at the midpoint of each month was determined for each of the pregnancy-to-estrus intervals shown (Table 7). The

number of pregnant cows was adjusted to a number equivalent to 21–25 days pregnant, by the use of the factors in the last column. For example, a cow pregnant 2–15 days was only 0.17 as likely to have estrus as one pregnant 21–25 days. As further shown, the number of postconception estruses observed was divided by the days in the month, to remove biases due to variable days in the months. Dividing this daily average by the number of pregnant cows (adjusted) gives a comparable percentage value for comparison of monthly variations.

Abortions would terminate some pregnancies before 250 days. However, error would be negligible, because abortions were reasonably well distributed among months and because of the low factors (0.04 and less) for cows pregnant over 100 days. Disposal of animals known to be pregnant would likewise limit observations for postconception estrus. There were 1,658 reproductive periods in the abortion and disposal categories which, if accounted for, would decrease the pregnant cows (adjusted) approximately 100 cows for the 12 mo. combined. There is no indication that more than ten of these would fall in any one month. As shown in Table 7, March, July, August, and October are low (18% below average). September is the only month not falling into a uniform cyclic pattern. This pattern shows decreasing values from January to March, increasing values from April to June, and generally low values from July to November. The percentage rates of the highest and lowest months compared with the average of all months are not significantly different at the 5% confidence limits and barely significant at the 30% level.

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INACTIVATION OF SPERMATOZOA BY LACTATE AND REACTIVATION WITH ALKALI

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SUMMARY

In two experiments, comparisons were made on the inactivation and reactivation by alkalinization of spermatozoa in two basic diluters: *(a)* YC, consisting of one part egg yolk and one part of a 2.9% solution of sodium citrate dihydrate, and *(b)* YGL, consisting of one part yolk, four parts of a 5% solution of glucose, and one part of different combinations of a 1.25% solution of lactic acid and a 0.6% solution of sodium hydroxide to have different pH levels. Survival of spermatozoa in the lactate diluents at 5° C. for seven days was not enhanced by the presence of sulfanilamide. Rather, complete inactivation of spermatozoa was observed after storage in YGL at pH 6.4, but not in YC at the same pH. Reactivation of spermatozoa after storage in the lactate medium can be accomplished by increasing pH to levels above 7.00. These spermatozoa are then capable of maintaining motility for at least 1 hr. at room temperature or for 24 hr. at 5° C., in spite of high pH. More work is needed to determine the value of inactivation with lactate in preservation of spermatozoa.

Up to the present time, the common method of prolonging viability and fertility of spermatozoa has been by cooling to above-freezing temperatures. With this method, the spermatozoa are immobilized and their metabolism is greatly reduced. A number of workers have reversibly immobilized spermatozoa by other means, at least for a short time. These means include addition of carbon dioxide *(12, 13)*, increase in hydrogen-ion concentration *(1, 3, 4, 10)*, and increase in concentration of potassium *(14)*. Milovanov and Khabibulin *(9)* found that spermatozoa could be immobilized by lactic acid or its salts accompanied by reduction of pH, and then could be reactivated by increase in temperature or alkalinization. They also mentioned that accumulation of carbon dioxide and lactic acid as metabolic products of spermatozoa is a factor in preservation at 5° C., as well as temperature per se.

In addition, a number of substances have been found to stimulate motility of spermatozoa after storage at temperatures above freezing. These include sodium chloride and glucose *(17)*, fresh seminal plasma *(15)*, and accessory gland secretions from a vasectomized male *(5)*. In addition, prostatic secretions *(6)* and alkali *(8)* stimulate motility of fresh semen.

Recently, fertility trials have demonstrated that some of these methods of inactivation and activation may be of value in artificial insemination. Rickard *et al. (11)* have reported a significant increase in nonreturn rate of semen after storage for two days above freezing. This was accomplished by adding sodium carbonate to semen in yolk-citrate before breeding cows. VanDemark

Received for publication September 26, 1957.

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and Sharma *(16)* recently bred a limited number of cows with spermatozoa held at room temperature for as long as seven days, in a medium containing sodium citrate, sodium bicarbonate, potassium chloride, glucose, egg yolk, and antibacterial agents, adjusted to about pH 6.35 with carbon dioxide. There was no evidence of reduction of fertility during that period.

During the course of investigation in the present authors' laboratory, of different buffers to be combined with egg yolk, some interesting observations have been made on inactivation of bull spermatozoa with lactate combined with reduction in pH and on reactivation with alkali. These observations and preliminary data have been reported previously in an abstract *(18)*.

Experiment I

Experimetal procedure. A comparison was made of survival of spermatozoa in six diluters: *(a)* One part yolk and one part of a 2.9% solution of sodium citrate dihydrate (YC); *(b)* YC but with 0.3% sulfanilamide; *(c)* two parts yolk, eight parts of a 5% solution of glucose, 0.9 part of a 1.25% solution of lactic acid, and 1.1 part of a 0.6% solution of sodium hydroxide (normal YGL). This preparation has a pH of approximately 6.8; *(d)* normal YGL with 0.3% sulfanilamide; *(e)* two parts yolk, eight parts 5% glucose, one part 1.25% lactic acid, and one part 0.6% NaOH (low YGL). This preparation has a pH of approximately 6.45; *(f)* low YGL with 0.3% sulfanilamide.

Dihydrostreptomycin sulfate was added to all treatments at a level of 500 γ per milliliter. For each ejaculate from a bull, 2 cc. of each diluter was placed in each of two small test-tubes. Semen was added to each tube to give a concentration of 15×10^6 spermatozoa per milliliter.

After storage at 5° C. for seven days, 0.35 ml. of a 1.3% solution of sodium bicarbonate was added to one of the tubes for each treatment. Motility observations were then made at 37° C. within 5 min. Two workers independently and without knowledge of the treatment estimated motility of spermatozoa in each sample. Duplicate estimations were averaged. Significance of differences between means was determined by analysis of variance.

Results. There were 13 ejaculates from ten bulls in the experiment. As can be seen (Table 1), there was a marked reactivation ($P<0.01$) of spermatozoa in both YGL diluters, especially in the absence of sufanilamide and in the diluter at low pH. There was little reactivation in YC ($P>0.05$). After addition of bicarbonate, the only significant differences among treatments were between the means for the two YGL diluters without sulfa on the one hand, and the smaller means for YC without sulfa and YGL-sulfa on the other. The interaction between sulfa and diluter treatments was highly significant ($P<0.01$).

Experiment II

Another experiment was conducted to determine if spermatozoa could be reactivated after being stored at 5° C. in yolk-citrate at a low pH, and if so-

TABLE 1

Average motility of spermatozoa and pH of diluters with and without reactivation with sodium bicarbonate when in yolk-citrate and yolk-glucose-lactate diluters with and without sulfanilamide

(Each datum is an average of 13 samples)

Storage period and poststorage treatment	Yolk-citrate		Yolk-glucose-lactate			
			Normal pH		Low pH	
	No sulfa	Sulfa	No sulfa	Sulfa	No sulfa	Sulfa
	Total motility *(%)*					
After seven days						
No $NaHCO_3$	34	47	45	42	13	18
With $NaHCO_3$	38	48	58	51	52	41
	Progressive motility *(%)*					
After seven days						
No $NaHCO_3$	24	33	23	24	6	8
With $NaHCO_3$	26	34	39	33	37	26
	pH					
Fresh	6.81	6.81	6.87	6.85	6.46	6.46
After seven days						
No $NaHCO_3$	6.63	6.61	6.49	6.51	6.17	6.23
With $NaHCO_3$	7.05	7.02	7.45	7.44	7.45	7.44

dium hydroxide would reactivate the spermatozoa as well as would sodium bicarbonate.

Experimental procedure. Three diluters were compared: YC with normal pH (average 6.79); YC with pH adjusted to approximately 6.4 with a 1.05% solution of citric acid; low YGL.

The only antibacterial agent added was dihydrostreptomycin sulfate at a level of 500 γ per milliliter.

On the seventh day of storage at 5° C., each diluter was subjected to five different treatments in separate, small test-tubes: 1. No additive. Motility examined after both seven and eight days of storage. 2. No additive. Motility examined after eight days of storage only. 3. Sodium bicarbonate added after seven days. 4. Sodium hydroxide added after seven days. 5. Respective diluter (0.35 ml. per milliliter of diluted semen) added after seven days.

Sodium hydroxide or sodium bicarbonate was added when the diluted semen was at a temperature of 5° C., and an attempt was made to have a final pH of 7.10.

After the seven-day storage period, and the addition of the alkali or respective diluter, Treatments I, III, IV, and V each received two additional subtreatments in separate test-tubes: *(a)* Held at 25° C. for 1 hr. and motility then observed; *(b)* held at 5° C. for 24 hr. and motility then observed.

General procedures and diluters were similar to those in Experiment I.

Results. In the experiment there were 13 ejaculates from eight bulls. The results are summarized (Table 2). There was only slight reactivation of spermatozoa upon addition of alkali to YC either at low or at normal pH. With all additive treatments, motility in YC at low pH was equal or superior to motility in YC at normal pH. None of these differences was significant, how-

TABLE 2

Motility of spermatozoa and pH of diluters after storage at 5° C. for seven days in yolk-citrate (YC) at pH 6.43 and 6.79 and in yolk-glucose-lactate (YGL) at pH 6.43

(Each datum is an average of 13 samples)

Treatment and diluter	Subtreatment number and additive				
	I None	II None	III $NaHCO_3$	IV NaOH	V Diluter
	Total motility *(%)*				
a) 1 hr. after additive					
YC (normal pH)	41	----	45	47	47
YC (low pH)	51	----	49	52	47
YGL (low pH)	2	----	34	45	1
b) 24 hr. after additive					
YC (normal pH)	39	39	34	41	42
YC (low pH)	40	46	39	43	47
YGL (low pH)	1	1	53	51	2
	Progressive motility *(%)*				
a) 1 hr. after additive					
YC (normal pH)	26	----	26	30	32
YC (low pH)	36	----	33	38	32
YGL (low pH)	0	----	26	33	0
b) 24 hr. after additive					
YC (normal pH)	22	24	18	22	26
YC (low pH)	36	36	30	29	33
YGL (low pH)	0	0	32	36	0
	pH				
b) 24 hr. after additive					
YC (normal pH)	6.68	6.70	7.12	7.06	6.67
YC (low pH)	6.38	6.34	7.05	7.13	6.37
YGL (low pH)	6.06	6.07	7.29	7.44	6.13
Fresh					
YC (normal pH)	6.79				
YC (low pH)	6.43				
YGL (low pH)	6.43				

ever. In YGL at low pH, reactivation was striking, with total motility increasing from 2 to 34 and 45% with $NaHCO_3$ and NaOH, respectively. Furthermore, in most instances motilities were greater after addition of sodium hydroxide than after addition of sodium bicarbonate, although the differences are not significant. These data indicate that the activation of spermatozoa is caused by increase in pH rather than by carbonate. The pH of the YC diluters was not raised to as high a level after addition of bicarbonate or sodium hydroxide as was the pH of the YGL diluters. The inference that might be drawn from this fact is that these differences might explain the differences in motilities. Detailed study of the individual observations had indicated, however, that comparatively little reactivation takes place in YC, no matter what pH levels are involved, and that reactivation takes place in YGL if pH is raised only to 7.00 or slightly higher. Although attempts were made to bring the pH of the different samples to the same pH level as the additives, difficulties were encountered because of the high buffering capacity of YC and the comparatively

low buffering capacity of YGL. The addition of fresh medium to the stored semen was ineffectual in reactivating spermatozoa.

Reactivated spermatozoa are capable of maintaining motility for at least 1 hr. at room temperature, or after storage at 5° C. for 24 hr., in spite of high pH.

DISCUSSION

After marked inactivation of spermatozoa was observed in YGL after storage, a number of samples were examined within ½ hr. after addition of spermatozoa to the different diluters. Only slight, if any, inactivation had taken place at that time. Rather complete inactivation was observable, however, after storage at 5° C. in YGL with low pH. This inactivation was not accomplished in yolk-citrate with a comparable reduction in pH. Inactivation in YGL with low pH was not enhanced by sulfanilamide. Possibly, its property of inhibiting metabolism of spermatozoa *(2, 7)* is harmful in this situation.

The inactivation of spermatozoa in YGL with low pH was more complete in Experiment II than in Experiment I. The authors have no explanation for this.

These observations with lactate emphasize the unreliability of motility alone as a criterion of viability of spermatozoa, when studying different substances as ingredients in diluents. Spermatozoa could often be adjudged immotile or as possessing poor motility when, in reality, they might be highly viable and could be reactivated by alkalinization.

More work is needed to determine optimum lactate and pH levels for inactivation of spermatozoa, optimum levels of pH for reactivation, the possible usefulness of this method of reactivation of spermatozoa when employed in conjunction with the techniques of VanDemark and Sharma *(16)* for preserving spermatozoa at room temperature, the fertility of spermatozoa after being subjected to those various treatments, and the necessity of reactivating spermatozoa before breeding cows.

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HIGH-LEVEL UREA FEEDING TO DAIRY CATTLE. I. EFFECT OF HIGH-LEVEL UREA FEEDING ON THE GROWTH AND METABOLISM OF GROWING DAIRY HEIFERS WITHOUT SULFUR SUPPLEMENTATION[1]

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SUMMARY

Twenty-four Holstein dairy heifers were used in a 150-day trial to study the effect of feeding grain rations containing 3.0, 5.0, and 7.0% urea upon growth, feed consumption, and metabolism. Ground corn cobs were fed as the sole source of roughage. As the levels of urea in the ration increased, the consumption of corn cobs decreased, although not significantly, and daily gains and feed efficiency decreased significantly. The average daily gain for heifers fed rations containing 3.0, 5.0, and 7.0% urea were 1.14, 0.85, and 0.64 lb. per day, respectively. The level of urea in the ration did not significantly affect nitrogen retention or blood levels of urea nitrogen, but as the intake of urea increased, blood serum levels of nonprotein nitrogen increased significantly and the levels of serum proteins significantly decreased. The possibility of a sulfur deficiency in a ration containing either 5.0 or 7.0% urea was discussed.

Numerous reports, as reviewed by Reid *(11)*, indicate that mature ruminants can utilize urea as a source of nitrogen for growth. In most of these experiments, urea did not compose more than 4% of the ration and did not supply more than 30-40% of the total nitrogen *(1, 2, 3, 6, 13)*. Reid *(11)* reviewed work at the Georgia station in which dairy heifers were fed rations containing 4.5% urea. The urea-fed heifers gained 1.0 lb. of body weight daily as compared with 1.4 lb. per day for heifers fed a ration in which cottonseed meal supplied most of the protein. Reid *(11)* stated that a difference in nutrient intake existed and that this could account for the differences in growth. In a second trial when the nutrient intake was balanced, the urea-fed heifers gained 1.4 lb. and the cottonseed-meal heifers 1.6 lb. per day. Urea supplied about 70% of the total protein equivalent of the ration during this latter trial. Recently, Iowa workers *(4)* formulated a beef cattle supplement containing 10% urea. Hart *et al. (7)* reported that calves (250-290 lb.) fed rations in which urea supplied 61 or 70% of the total nitrogen had diuresis.

The purpose of this study was to determine the maximum level of urea that could be efficiently utilized by growing dairy heifers.

EXPERIMENTAL PROCEDURE

Twenty-four Holstein dairy heifers were selected and divided into three groups, after considering body weight, age, and condition, for use in a 150-day

Received for publication May 7, 1957.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2072.

feeding trial to evaluate high-level urea feeding. This study was conducted in two trials. Nine heifers were used in Trial I and 15 heifers were used in Trial II.

All of the heifers were fed ground corn cobs as the sole roughage at an approximate rate of 2 lb. per 100 lb. of body weight daily. Adjustments were made in feeding during the experiment, but the amount of feed offered was always the same for each group. Each group was fed one of the experimental supplements (Table 1) at a rate to equal Morrison's *(10)* minimum protein standards and 110% of the minimum T.D.N. standards.

TABLE 1

Composition of experimental cattle supplements

Ingredients	Rations 1	2	3	Corn cobs
		(lb.)		
Ground yellow corn	589.5	719.5	849.5	
Soybeal oil meal	300.0	150.0	.....	
Urea	30.0	50.0	70.0	
Dicalcium phosphate	60.0	60.0	60.0	
Trace salt[a]	20.0	20.0	20.0	
Vitamin A and D concentrate[b]	0.5	0.5	0.5	
Total	1,000.0	1,000.0	1,000.0	
		Chemical analysis (%)		
Dry matter	88.6	88.9	88.6	85.4
Crude protein	26.2	25.8	25.1	2.6
Crude fiber	2.8	2.2	2.0	28.6
Ether extract	2.7	3.2	3.0	0.5
N-free extract	48.0	49.7	50.5	52.4
Ash	8.9	8.0	8.0	1.3
T.D.N.[c]	70.6	69.3	68.0	.....
Nitrogen supplied by urea	30.0	50.0	70.0	.....
Sulfur	0.171	0.141	0.112	0.027

[a] Salt plus iodine and cobalt.
[b] 10,000 I.U. of vitamin A and 1,250 I.U. of vitamin D per g. of concentrate.
[c] Morrison *(10)*.

The heifers were weighed for three consecutive days, at the beginning of the experiment and at 30-day intervals thereafter. Nitrogen balance trials were conducted on each heifer once per month, using a six-day collection period. Blood samples were taken prior to the beginning of the experiments and at 2-wk. intervals thereafter, at approximately the same time each day, and analyzed for blood urea nitrogen, serum nonprotein nitrogen, and total serum nitrogen.

The composition of the experimental supplements, as well as the corn cobs, is shown (Table 1). The major difference in the three experimental grain mixtures was in the amount of nitrogen supplied by urea. In Rations 1, 2, and 3, urea supplied 30, 50 and 70% of the total nitrogen, respectively.

RESULTS AND DISCUSSION

Data showing effects of different levels of urea feeding on feed consumption and body weight changes are presented (Table 2). No significant differences existed between groups in respect to feed consumption, but as the urea percentage

TABLE 2

Effect of high-level urea feeding on the average feed consumption, feed efficiency, and body weight gains of growing dairy heifers

Group	Feed	Daily feed	Initial weight	Final weight	Daily gain	Feed per lb. grain
(No.)				*(lb.)*		
1	Corn cobs Supplement	13.0 4.1	590	762	1.15	15.0
2	Corn cobs Supplement	12.6 4.1	594	722	0.85	19.6
3	Corn cobs Supplement	12.1 4.0	591	687	0.64	25.2
L.S.D.—1%		N.S.			0.18	N.S.
L.D.D.—5%		N.S.				7.2

of the rations increased there was a tendency for the consumption of corn cobs to decrease. This was particularly true during Trial I, with heifers fed Ration 3. The heifers on this ration exhibited periods of off-feed and severe diarrhea for about the first 3.5 mo. of the trial. After this time, the heifers ate normally. Even though the heifers fed Ration 3 consumed slightly less feed than heifers on Ration 1 during Trial II, the nature of this off-feed condition was not as severe as in Trial I, nor did the heifers exhibit the diarrhea condition. Heifers fed Rations 1, 2, and 3 consumed 56, 93, and 127 g. of urea per day, respectively, or 8.3, 14.1, and 19.8 g. of urea per 100 lb. of body weight.

As is shown (Table 2), as the urea content of the rations increased, the daily rate of gain and feed efficiency of the heifers decreased significantly. This decreased rate of growth was evident throughout the experiment for the heifers fed high levels of urea nitrogen. Some differences between trials in respect to growth rate were observed, but these differences did not appear to be significant. As shown in Table 1, the grain rations were not supplemented with sulfur. It should be noted that as the urea content of the rations increased, the sulfur content decreased. Several workers, including Thomas *et al (12),* observed that rations fed to sheep containing urea require sulfur supplementation. Jones and Haag *(8)* reported inconclusive results which showed that sodium sulfate may improve the utilization of urea in a ration containing 3% urea and with an over-all sulfur content of 0.13%. Jones *et al. (9)* and Davis *et al. (5)* failed to confirm this observation. The type of ration fed and its original sulfur content, however, would be important factors in determining whether cattle need sulfur supplementation.

If the sulfur content of Rations 2 and 3 had been equal to that of Ration 1, it is quite possible that these heifers would have shown improved growth rates; possibly equal to those of the heifers fed Ration 1. Studies are now in progress to test this hypothesis.

The level of urea nitrogen intake had very little effect on the retention of nitrogen or the efficiency of nitrogen utilization (Table 3). This observation in relation to decreased growth rates with high-level urea feeding is difficult to

TABLE 3

Effect of high-level urea feeding on the average nitrogen retention of growing dairy heifers

	Ration No.			1% L.S.D.
	1	2	3	
Daily nitrogen intake (*g.*)	100.5	99.5	95.9	N.S.
Daily nitrogen excretion (*g.*)	54.1	49.3	50.8	N.S.
Daily nitrogen retention (*g.*)	46.4	50.2	45.1	N.S.
Retention of intake (%)	46.2	50.5	47.0	N.S.

explain. Digestion trials were not conducted, but calculations based upon the total intake and excretion of dry matter (feces plus urine) failed to show any great differences among rations. Heifers fed Rations 1, 2, and 3 utilized 61.5, 61.7, and 59.4%, respectively, of the dry matter intake. These differences among rations were not statistically different.

The effect of high-level urea feeding on various blood nitrogen and protein constituents is shown (Table 4). The high intakes of urea nitrogen did not have

TABLE 4

Effect of high-level urea feeding on the blood levels of urea nitrogen, serum nonprotein nitrogen, and serum protein of growing dairy heifers

Weeks on experiment	Group No.								
	1	2	3	1	2	3	1	2	3
	Urea nitrogen			Nonprotein nitrogen			Serum protein		
	(*mg. %*)						(%)		
0	16.4	16.7	16.8	28.0	27.5	26.7	7.09	7.08	6.70
2	8.2	15.2	11.8	22.1	24.7	24.8	6.87	6.67	6.49
4	6.1	12.1	10.7	21.4	25.0	26.4	6.71	6.74	6.38
6	5.9	11.0	11.7	20.0	25.9	27.3	6.94	6.87	6.42
8	7.2	12.2	11.7	22.6	24.2	25.8	6.65	6.66	6.49
10	9.2	9.7	8.4	21.8	24.6	24.0	6.55	6.49	6.09
12	9.4	11.1	12.0	23.7	25.1	27.9	7.05	6.75	6.55
14	9.0	12.1	9.5	22.9	23.9	25.0	6.86	6.57	6.33
16	7.4	6.9	9.5	19.7	21.6	24.6	6.71	6.71	6.30
18	5.5	7.0	5.8	22.9	21.9	19.8	7.39	7.15	6.60
20	8.7	7.1	7.2	20.8	21.8	17.0	7.02	6.69	6.19
Average	7.5	9.3	9.5	21.8	23.9	24.3	6.87	6.73	6.38
L.S.D.—1%		N.S.			1.8			0.07	

a significant effect on blood urea nitrogen, although these differences did approach significance. The concentration of blood urea nitrogen showed a tendency to increase as the intake of urea nitrogen increased. Similar significant trends existed for serum nonprotein nitrogen. The concentration of serum protein decreased significantly as the intake of urea nitrogen increased. These phenomena apparently indicated a disturbance of protein metabolism when high levels of urea were fed.

ACKNOWLEDGMENTS

The authors wish to express appreciation to Dr. D. F. Green of Allied Chemical Dye Corporation, New York City, for supplying the urea used in this study.

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COTTONSEED MEALS AS THE PRIMARY PROTEIN SUPPLEMENT IN CONCENTRATE FEEDS FOR YOUNG CALVES[1,2]

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SUMMARY

Responses of calves to starters containing cottonseed meals produced by different methods and containing different concentrations of free gossypol were investigated. Starters containing hydraulic-pressed cottonseed meal at levels of 40 and 60% were compared with similar starters containing a special screw-pressed cottonseed meal. Concentrations of free gossypol in the starters were 0.107, 0.071, 0.035, and 0.023%, respectively. Corresponding mortality rates among calves consuming these starters ad libitum were 100, 50, 75, and 0%. There was a positive correlation between free-gossypol intake by the calves up to 48 days of age and subsequent mortality. The maximum safe intake of free gossypol during this period was approximately 140 mg. per cwt. per day. Comparisons of calf starters containing 40 and 60% of degossypolized (0.029% free gossypol) cottonseed meal with starters containing corresponding levels of solvent-extracted soybean oil meal and with a starter having a blend, 40% of the former and 20% of the latter, revealed no significant difference in weight gains of calves during a 120-day period. Weight increases in these comparisons were similar to those from the starter containing the special screw-pressed cottonseed meal. The observations substantiate the prevalent view that young calves are highly sensitive to gossypol toxicity, but the free-gossypol content of the cottonseed meals produced by improved processes was sufficiently low to permit the use of these meals as the primary source of protein in practical calf starters.

Cottonseed meal is recognized as an important source of protein in the diets of cattle older than 3 or 4 mo. For younger cattle, however, 20% cottonseed meal in the concentrate mixture generally has been regarded as the maximum safe level. This is based on the observation that young calves are sensitive to cottonseed meal poisoning *(4, 7, 8, 12, 18)*, which apparently is related to the gossypol content of the meal. Though feeding cottonseed meal ad libitum in conjunction with good-quality hay and liberal amounts of milk to calves as young as 30 days of age evoked no adverse responses *(2, 8, 11)*, daily feeding of from 0.11 to 0.40 lb. of cottonseed meal dispersed in milk caused the death of 27 of 28 young experimental calves within a period of from 33 to 73 days *(8)* after initiation of feeding. The variability in responses of calves to cottonseed meal feeding under different dietary conditions raises

Received for publication August 2, 1957.

[1] Approved by the Director of Research for publication as Paper No. 833 in the Journal Series.

[2] The data in this paper are from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science.

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a question of whether or not gossypol intake is the primary etiological factor involved in the difficulties observed. Recent advances in cottonseed processing, resulting in the production of meals low in free gossypol and high in nutritive value for nonruminants, provide means of reappraising the relation of gossypol to the value of cottonseed meals in the diets of calves.

Thus, the principal purpose of the work reported herein was to obtain information on the responses of young calves to concentrate feeds (starters) containing different kinds and amounts of cottonseed meals and different levels of free gossypol.

EXPERIMENTAL PROCEDURES AND RESULTS

The study consisted of two feeding trials, I and II, in which cottonseed meals produced by improved processing methods were compared with conventional types of oil-seed meals, either cottonseed or soybean.

Trial I

Procedure. In this trial, a special screw-pressed meal (low in free gossypol) was compared with a hydraulic-pressed meal (high in free gossypol), typical of the type commonly produced in the past. Comparisons of these two meals were made at levels of 40 and 60% in Starters 1 through 4 (Table 1). The resulting percentage of free gossypol in these mixtures ranged from a high of 0.107 to a low of 0.023 (Table 2). Minimization of differences in amounts of protein in the four starters was effected through incorporation of soybean oil meal in those containing the lower levels of cottonseed meal. The concentrations of crude protein thus obtained in the starters were between 28.3 and 29.4%.

Sixteen male calves, three replications of Holsteins and one of Jerseys, were

TABLE 1

Ingredient composition of starters used in testing the relative nutritional value of different cottonseed meals

Ingredients	Proportions of ingredients used								
	Starters of Trial I				Starters of Trial II				
	1	2	3	4	5	6	7	8	9
	(%)								
Cottonseed meal (hydraulic, 0.179% free gossypol)[a]	60.0	40.0	.....	.....	.....	.....	.....	.....	.....
Cottonseed meal (expeller, 0.059% free gossypol)[b]	.....	.....	60.0	40.0	.....	.....	.....	.....	.....
Cottonseed meal (degossypolized, 0.029% free gossypol)[c]	.....	.....	.....	.....	60.0	40.0	.....	.....	40.0
Soybean oil meal	.....	15.0	.....	15.0	.....	.....	60.0	40.0	20.0
Ground yellow corn	18.5	21.0	18.5	21.0	18.5	28.5	18.5	28.5	18.5
Rolled oats	18.5	21.0	18.5	21.0	18.5	28.5	18.5	28.5	18.5
Steamed bonemeal	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Common salt	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Provided by National Cottonseed Products Association, Dallas, Texas.

[b] Special screw-pressed, low-temperature–cooked meal supplied by USDA Southern Regional Research Laboratory, New Orleans, La.

[c] Aromatic-amine treated. Produced and supplied by the Buckeye Cotton Oil Co., Cincinnati, Ohio.

assigned at random to the starters. The animals were received at three days of age and were continued on experiment either until death ensued or until the scheduled terminal stage of 180 days of age was reached. The calves were retained throughout the trial in individual pens bedded with wood shavings.

TABLE 2

Periods of survival of calves receiving starters containing different sources and levels of cottonseed meals and having different concentrations of free gossypol, Trial I

Starters		Experimental calves		Av. free-gossypol intake cwt/day			
				By periods (days)[b]			
No.	Free gossypol	Identification[a]	Age at death	[c]–48	49–80	81–120	121–180
	(%)		*(days)*	*(mg.)*			
1	0.107	H-57	90	402	1,029	.....	.....
		H-60	48	641	.....	.....	.....
		H-69	120	500	1,208	747	.....
		J-67	120	325	1,199	825	.....
2	0.071	H-56	89	435	634	.....	.....
		H-62	Survived	142	477	712	721
		H-68	54	351	.....	.....	.....
		J-58	Survived	473	754	824	812
3	0.035	H-55	80	141	333	.....	.....
		H-59	132	162	238	191	.....
		H-66	Survived	113	192	321	310
		J-63	81	227	419	.....	.....
4	0.023	H-54	Survived	55	257	216	200
		H-64	Survived	40	105	233	215
		H-65	Survived	76	173	240	197
		J-70	Survived	72	257	265	249

[a] H = Holstein and J = Jersey. Sequence in each group corresponds to replications.
[b] Periods relate to initiation of a sequence of deaths: Two calves died between 48 and 56 days, four between 80 and 90 days, and three between 120 and 132 days.
[c] Rates in first period were calculated on the basis of number of days after calves began eating starters, consumption of which was initiated from four to 11 days of age.

The dietary regimen was typical of that employed in conventional calf husbandry. Fresh whole milk was fed via a nipple pail, at the daily rate of 10 lb. per cwt. of the calf, during the first 30 days. Thereafter, the allotment was reduced daily through the 36th day, after which milk feeding was discontinued. During the milk-feeding period, calves received 10,000 I.U. of vitamin A and 2,700 I.U. of vitamin D daily. Good-quality alfalfa hay was offered free choice to the calves throughout the time they were on experiment. After the initial four to 12 days of the experimental period, one of the aforementioned starters was fed to each calf in amounts commensurate with its daily consumption.

Responses of the calves to the experimental diets were indicated by starter consumption, by changes in body weight and in clinical health, and by post-mortem lesions. Consumption of hay was not recorded. Each calf was weighed when it was assigned to the experiment and at weekly intervals thereafter. All animals were examined twice daily to note any evidence of abnormalities. Special attention was given to the recording of the incidence and severity of

diarrhea. Calves that died during the trials were autopsied, and samples of liver were collected from representative individuals for histological examination.

Results. Since mortality was high among the calves in Trial I, emphasis is given primarily to the relationship of diets to health and survival, and secondarily to starter consumption and growth.

(a) Mortality rate. The death rate was strikingly high (Table 2), particularly in the group receiving Starter 1, which group was followed in decreasing order by those fed Starters 3 and 2, respectively. The deaths, which occurred between 48 and 132 days of age, inclusively, were distributed as indicated (footnote *b,* Table 2). There was a significantly ($P \leqslant 0.05$) positive correlation between the free-gossypol intake during the initial period, to 48 days of age of the calves, and subsequent mortality. With the notable exception of H-62 and J-58 (Starter 2), all calves that ingested free gossypol at an average rate of more than 140 mg. per day per cwt. of animal during this initial consumption period died. After this first stage, the relationship between free-gossypol intake and death incidence was more variable.

(b) Symptoms. Of the calves that died, the most common antemortem symptom was the development of an erratic appetite from 1 to 2 wk. before death. During the terminal two days, abdominal pains and dyspnea were evidenced frequently. In two individuals, however, there were no premonitory symptoms prior to death. Incidences of gross postmortem lesions (Table 3) show that blood abnormally bright-red in color and extremely slow in coagulation, hydroperitoneum, and hydrothorax were common in almost all the calves. Other lesions varied not only in incidence but in severity. Histological studies of liver tissues from representative calves revealed fatty degeneration.

TABLE 3

Summary of autopsy findings in nine calves that died during Trial I

Macroscopic lesions	Incidence	
	No.	Percentage
Blood, bright-red and incompletely coagulated	9	100
Serous fluids in abdominal cavity	9	100
Serous fluids in thoracic cavity	8	89
Enteritis	7	78
Lungs, congested	6	67
Abomasitis	6	67
Subcutaneous edema	6	67
Kidneys, edematous	5	56
Suggillations of the arterio-ventricular valves of the heart	5	56
Intestines, edematous	4	44
Epicardial fat, petechiated	3	33
Liver, enlarged and hardened	2	22
Lungs, hepatization of parts	2	22
Epicardial fat, edematous	2	22
Mesentery membrane, hemorrhagic areas	2	22
Spleen, petechiated	2	22

Among the survivors, unthrifty appearance, as manifested by harsh coats and paunchiness, was common to all. Incidence of diarrhea was low and inter-

mittent except in Calf J-58, in which scours were chronic after about the 50th day of the trial.

(c) Starter consumption and weight gains. Starter intakes and resulting gains (Table 4) reveal considerable intragroup variation, as well as intergroup differences. The time necessary for the daily intake to increase to 3 lb. was

TABLE 4

Rates of consumption of starters having different sources and levels of cottonseed meals, and associated rates of gains in weight of calves, Trial I

Starter No.	Calf No.[a]	Rates of starter consumption: Began consumption 3 lb/day[b]	Rates of starter consumption: By periods (days)[c] d–48	49–80	81–120	121–180	Rates of gain: By periods (days)[c] d–48	49–80	81–120	121–180
		(day)	*(lb/day/cwt)*				*(lb/day)*			
1	H-57	45	0.83	2.12	Dead	.....	0.9	0.8	Dead	.....
	H-60	40	1.23	Dead	.....	.....	1.2	Dead	.....	.....
	H-69	53	1.03	2.49	1.54	Dead	1.0	1.3	0.8	Dead
	J-67	53	0.67	2.47	1.70	Dead	0.7	1.0	0.2	Dead
2	H-56	39	1.35	1.97	Dead	.....	1.3	1.1	Dead	.....
	H-62	71	0.44	1.48	2.21	2.24	0.6	0.7	1.3	2.1
	H-68	(e)	1.09	Dead	.....	.....	1.0	Dead	.....	.....
	J-58	40	1.47	2.34	2.56	2.52	1.0	1.0	1.2	2.0
3	H-55	48	0.89	2.10	Dead	.....	1.3	1.8	Dead	.....
	H-59	41	1.02	1.50	1.20	Dead	1.0	0.8	0.7	Dead
	H-66	82	0.71	1.21	2.02	1.95	1.3	1.1	1.3	2.5
	J-63	41	1.43	2.64	Dead	.....	0.9	0.5	Dead	.....
4	H-54	49	0.53	2.46	2.07	1.92	0.4	1.3	0.8	1.5
	H-64	83	0.38	1.01	2.23	2.06	0.8	1.2	1.9	2.8
	H-65	57	0.60	1.66	2.29	1.89	1.1	1.5	2.5	2.5
	J-70	80	0.93	2.46	2.54	2.39	0.8	1.3	0.9	2.0

[a] H = Holstein and J = Jersey; sequence in each group corresponds to replications.
[b] Initial age when consumption of starter increased to the rate of 3 lb/day for two successive days.
[c] Periods related to survival of calves (see Table 2).
[d] Calculations for the initial period based on days after calves began consuming starters.
[e] Calf died before daily consumption reached 3 lb.

extremely variable, ranging from 39 to 83 days. Of the groups in which death occurred, the survivors, except J-58 of Group 2, were slow in reaching the 3-lb.-per-day level. Generally, there was decreased consumption of starters and either a retardation in the rate of gain or a slight loss of weight in the terminal stages of the lives of calves that died during the trial. Among the surviving calves in all dietary categories, the maximum rate of starter intake occurred, for the most part, between 81 and 120 days; whereas, the most rapid gains were during the concluding 60 days of the trial. Gains did not reveal any distinct differences attributable to diet.

Trial II

Procedure. Although results from Trial I suggested that the primary factor in the cottonseed-meal diets affecting the responses of young calves was the free-gossypol intake, the quality of the protein, apart from the gossypol, possibly was involved also. Thus, in Trial II a commercial degossypolized cottonseed

meal was compared with a solvent-extracted soybean oil meal and with a mixture of these two meals. The percentages of the respective meals incorporated in the experimental Starters 5 through 9 are shown (Table 1). Since the crude protein concentrations were not equated in these starters, those having 40% oil meal contained 22.5% protein and those having 60%, 29.4% protein.

Four complete replications of calves, two of Holsteins, one of Ayrshires, and one of Jerseys, were assigned at random to the starters. An incomplete replication of Guernseys also was represented in each dietary group except 6 and 9. The calves were under experimental conditions from the age of three to 120 days. Feeding and management were as described for calves in Trial I.

Results. The primary criteria of responses to the diets studied in this trial were apparent health, starter ingestion, and weight gains.

(a) General health. Though all calves survived, the predominance of rough coats and of paunchiness, as noted in Trial I, was evident in all dietary groups throughout Trial II. The incidence and the severity of these abnormalities were about equal among the various dietary groups. Occasional diarrhea occurred in calves of all regimens but apparently did not affect any animal severely.

(b) Starter consumption and weight gains. A summary of the quantities of starters consumed and the gains in weights of calves in the various dietary groups (Table 5) reveals no significant differences. Neither the starter intake nor the weight increase was affected significantly, either by the kind of oil meal or by its concentration in the mixture.

TABLE 5

Intakes and gains of calves fed starters containing various oil meals present in different concentrations in the diet, Trial II[a]

Starters			Total starter consumption/calf		Total gain in wt/calf	
Oil meal used						
Kind	Concentration	No.	Av.	Range	Av.	Range
	(%)		*(lb.)*			
Degossypolized cottonseed meal	60	5	273	221–343	116	87–163
	40	6	289	220–363	135	99–171
	Av.	....	280	.....	124	.....
Soybean oil meal	60	7	294	211–375	142	97–165
	40	8	260	182–353	140	108–188
	Av.	....	277	.....	141	.....
Cottonseed and soybean	40 C. and 20 S.	9	289	141–344	133	96–174

[a] Duration of trial, three to 120 days of age.

DISCUSSION

Responses of calves to starters containing different concentrations of free gossypol substantiate the generally accepted view that the young calf is more susceptible to gossypol toxicity than is the older calf. On the basis of the relationship of age to the tolerance of free-gossypol ingestion, the responses correspond to the functional development of the rumen. Conceivably, free gossypol may be

diluted in the rumino-reticular contents and either complexed with rumen constituents or disintegrated to the extent that the rate and the degree of absorption of free gossypol are reduced, resulting in tolerable systemic concentrations.

Evidence that the lethality of gossypol is closely related to its concentration in the blood was indicated by the immediate manifestations of toxicity when gossypol was injected directly into the blood stream of rabbits *(10)*. Therefore, a logical assumption is that conditions retarding absorption would tend to reduce the hazards of diets containing free gossypol.

The individual differences in responses to free-gossypol intake, Trial I, are in harmony with observations by Leighton *et al. (9)*. Their findings indicated further that Jerseys may be more tolerant than Holsteins to gossypol, but the data from Trial I are inadequate to elucidate this point. Though many factors may contribute, either directly or indirectly, to variability in reactions of individuals to ingested free gossypol, incidence of scours may be one of the conditions commonly involved. The possibility of scouring interfering with gossypol absorption is indicated by the apparent immunity of a diarrhetic calf, J-58, which consumed relatively large amounts of free gossypol.

Indefiniteness of available information on the role of different factors affecting responses of calves to free-gossypol consumption renders difficult the estimation of the maximum intake tolerated. Results from Trial I indicate that a mean daily intake of free gossypol exceeding 140 mg. per cwt. during the initial starter-consuming period of from 5 to 6 wk. in the first 7 wk. of the life of the calf is likely to be lethal. In Trial I, survival was 100% only from the mixture having the lowest level, 0.023%, of free gossypol. The fact that both milk and hay also were included in the diets of the calves during the first 5 wk. of the trials suggests that the safe concentration of free gossypol in the total feed ingested probably was near 0.01% level tolerated by fattening-growing pigs *(5)*.

Though the concentration of free gossypol in the starters was related positively to the mortality of the calves, the gossypol intake apparently had little effect on the appetite and the growth response of the calves prior to the manifestation of clinical symptoms of gossypol toxicity. The syndrome was typical, for the most part, of that usually reported *(3, 8, 9, 16-18)*. One possible exceptional observation was the retardation of the coagulation of the blood of calves autopsied. This characteristic of the blood suggested that large intakes of free gossypol may interfere with coagulation. Harms and Holley *(6)* reported that gossypol produced hypoprothrombinemia in rabbits and pigs. Exploratory tests of the blood from calves in Trial I indicated a tendency for the higher intakes of free gossypol to increase the clotting time. However, further investigations are necessary to clarify this suggested tendency.

Recent studies with swine *(5, 15)* and rats *(1, 14)* indicate that the quality of proteins and their concentrations in the diets may modify the effects of ingested free gossypol. Comparisons of survival in dietary groups 2 and 3 of Trial I indicated a possible advantage of a blend of cottonseed meal and soybean

oil meal over cottonseed meal as the only supplement. This suggested difference, however, was not reflected in the comparative growth responses from starters containing degossypolized cottonseed meal, soybean oil meal, and a blend of the two meals, Trial II. Free-gossypol intakes in this trial possibly were too low and the levels of protein too high to provide a crucial test of the relationship of dietary proteins to the gossypol syndrome in the young calf.

The similarity of the gains among calves in all dietary groups of Trial II indicates that the degossypolized cottonseed meal was as satisfactory as the soybean oil meal in providing the primary source of protein in calf starters. This is in accord with the findings of Rusoff *et al. (12)* when antibiotics were not fed, but when their calf diets were supplemented with aureomycin, the superiority of soybean oil meal was indicated. In Trial I, the weight increases (average 140 lb. in 120 days) of calves receiving the starter containing the special screw-pressed meal at the 40% level were within the range of gains noted in dietary groups of Trial II. Thus, the data reported herein indicate that both the new cottonseed meals, the special screw-pressed and the degossypolized, would be acceptable as primary sources of protein, even at concentrations greater than commonly used, in starters for young calves.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of C. D. Grinnells in providing facilities, and of J. C. Osborne for autopsies and histological examinations; and the cooperation of the National Cottonseed Products Association, Dallas, Texas; the USDA Southern Regional Laboratory, New Orleans, Louisiana, and the Buckeye Cotton Oil Company, Cincinnati, Ohio, for supplying the various cottonseed meals studied.

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STUDIES ON THE EXPERIMENTAL PRODUCTION AND PREVENTION OF BLOAT IN CATTLE. II. THE INFLUENCE OF DEHYDRATION ON THE BLOAT-PRODUCING ABILITY OF ALFALFA[1]

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SUMMARY

This study, in comparing the bloat-producing ability of alfalfa before and after commercial dehydration, demonstrates that this process greatly reduces the ability of alfalfa to produce bloat. A coincident study was made of the effect of fresh and dehydrated alfalfa on ruminal motility and eructation in one cow. In the first trial, fresh alfalfa was assayed for three consecutive days, following a four-day preliminary period, using eight bloat-susceptible cattle. Alfalfa, collected from the same field on the second day of the assay period, was dehydrated for the subsequent comparative trials. The fresh alfalfa was fed ad libitum for 2-hr. periods, at 8 A.M. and at 1 P.M. Alfalfa consumption for each cow was determined. The degree of bloat was estimated with a tympanometer. The same procedure was followed in assessing the dehydrated alfalfa, except that each cow's intake was limited on the basis of dry matter to the amount of fresh alfalfa consumed previously. The average, maximal, intrarumen gas pressures attained were 27 mm. Hg above atmospheric pressure with fresh alfalfa, and < 5 mm. Hg after receiving dehydrated alfalfa. It is suggested that a partial explanation for this reduction of bloat associated with dehydration may be a denaturation of water-soluble plant protein.

EXPERIMENTAL PROCEDURE

Influence of fresh alfalfa on bloat. Thirteen dry Jersey cows were placed in a dry lot and fed individually, in stanchions, a known amount of fresh alfalfa ad libitum for 2 hr. at 8 A.M. and again at 1 P.M. The alfalfa was harvested in the prebloom stage of maturity from the same field at approximately 7 A.M. each morning with a Gehl standard dy-wheel type forage harvester. The upper 4 to 6 in. of the alfalfa plants were collected and chopped. Samples were taken at each cutting for moisture analysis. The fresh alfalfa contained, on the average, 15.2% dry matter. At the end of each 2-hr. feeding period, alfalfa consumption was determined for each animal and they were released from the stanchions and allowed free access to water. Supplemental feed was not given. Thirty minutes after the start of each feeding period, and at 15-min. intervals thereafter, until intrarumen gas pressure returned to prefeeding levels, the degree of bloat was estimated with a tympanometer (8) when the rumen was not contracting. When the intrarumen pressure attained 50 mm. Hg above atmospheric pressure, bloat was considered dangerous and treatment was initiated. This consisted of the intrarumen administration of 100 ml. of vegetable oil (Wesson).

Received for publication March 12, 1957.

[1] In cooperation with the Animal Disease and Parasite Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

The total experimental period lasted seven days, including a preliminary period of four days (July 25 through July 28). At the end of the preliminary period, the cows had attained a fairly stable alfalfa intake, and moderate bloat occurred in most of the animals. At this time, the five animals least susceptible to bloat were discarded.

The bloat-producing ability of the fresh alfalfa was assessed during the last three days of the experimental period (July 29 through July 31). On July 30, several tons of alfalfa were harvested and then dehydrated and pelleted for the subsequent comparative trial on the bloat-producing ability of this material.

Influence of dehydrated, pelleted alfalfa on bloat. The experimental procedure followed in this phase of the investigation was nearly identical to that outlined above. However, the dehydrated alfalfa intake of each cow during the 2-hr. feeding periods was limited, on an oven-dry basis, to the amounts of fresh material consumed during the preceding trial. Thus, the dry matter intake for each animal at each feeding period in both trials was nearly the same. The experimental period lasted seven days, consisting of a four-day preliminary period (August 5 through August 8) and a three-day period (August 9 through August 11), when the bloat-producing ability of dehydrated alfalfa was evaluated and compared with that observed on fresh alfalfa. Again, the degree of bloat was estimated using the tympanometer *(8)*.

Influence of fresh vs. dehydrated alfalfa on ruminal motility and eructation. In addition to the trials just outlined, a coincident study was made of the influence of both the fresh and dehydrated alfalfas on ruminal motility and eructation. One dry Jersey cow (897) was used in this phase of the investigation. The preparation of the animal and the apparatus employed have been described in previous papers *(1, 4)*.

Cow 897 was subjected to the same feeding procedure as in the trials just described, except that the individual feeding periods were reduced to 1 hr. when fresh alfalfa was fed and to ½ hr. during the dehydrated alfalfa trials, to allow a measurement of eructation as soon as possible after the entrance of food into the rumen. Again, the amount of dehydrated alfalfa fed was limited to the amount of fresh material consumed (on the basis of dry matter). However, the former was consumed more rapidly and the feeding periods could be reduced to ½ hr. Six trials were conducted using Cow 897, in addition to the four-day preliminary periods, three trials using the fresh alfalfa harvested on July 29, 30, and 31, and three trials using the dehydrated, pelleted alfalfa harvested on July 30. The average dry matter consumption during the six trials was 4.6 lb. The total daily alfalfa intake was limited to 9.2 lb. of dry matter.

RESULTS

Influence of fresh vs. dehydrated alfalfa on bloat. The maximal intrarumen gas pressures attained, as well as the dry matter intakes, for each animal fed

TABLE 1

Comparative effects of freshly chopped alfalfa and of ground, dehydrated, pelleted alfalfa upon the maximal intrarumen gas pressure of dairy cattle

Cow No.	Fresh alfalfa			Dehydrated alfalfa		
	Date	Dry matter intake	Maximum intrarumen pressure	Date	Dry matter intake	Maximum intrarumen pressure
	(1956)	*(lb.)*	*(mm. Hg)*	*(1956)*	*(lb.)*	*(mm. Hg)*
4	7–29	8.2	5	8–9	7.5	<5
	7–30	10.0	11	8–10	7.5	<5
	7–31	7.8	27	8–11	9.3	<5
47	7–29	4.5	14	8–9	7.5	<5
	7–30	7.8	21	8–10	6.5	6
	7–31	7.9	23	8–11	7.5	6
50	7–29	4.9	28	8–9	3.7	<5
	7–30	8.0	19	8–10	0.9	<5
	7–31	5.8	40	8–11	4.7	<5
54	7–29	4.2	39	8–9	7.5	<5
	7–30	7.2	32	8–10	7.5	<5
	7–31	5.9	50[a]	8–11	8.4	<5
58	7–29	6.1	36	8–9	7.5	7
	7–30	6.5	27	8–10	7.5	7
	7–31	7.3	40	8–11	10.3	<5
70	7–29	4.4	16	8–9	6.5	<5
	7–30	6.3	24	8–10	3.7	<5
	7–31	8.0	23	8–11	7.5	<5
79	7–29	4.2	36	8–9	7.5	6
	7–30	8.6	12	8–10	7.5	<5
	7–31	7.2	32	8–11	5.6	<5
112	7–29	3.5	29	8–9	2.8	6
	7–30	6.2	26	8–10	7.5	<5
	7–31	5.5	36	8–11	4.7	<5

[a] Treated for bloat by administration of 100 ml. of vegetable oil via stomach tube.

alfalfa before and after dehydration appear in Table 1. The average maximal intrarumen pressure was much greater when fresh alfalfa was fed (27 mm. Hg as compared with less than 5 mm. Hg above atmospheric pressure on the dehydrated material). In most cases, the intrarumen pressures reached a maximum from 90 to 120 min. after the start of the feeding period. Only the data obtained at the morning feeding periods are included in Table 1. Although alfalfa consumption was approximately the same during the afternoon, bloat was not so severe, in most animals, as in the morning. In some animals the consumption of dehydrated alfalfa for a particular day was considerably less, on the basis of dry matter (Cow 50, July 30, for example), than the previous intake of fresh alfalfa, because of the relative unpalatability of the former. However, the average intakes were nearly equivalent—6.50 lb. of fresh alfalfa, 6.55 lb. of dehydrated alfalfa.

When fresh alfalfa was fed, one of the animals (Cow 54) bloated rather severely on six occasions. On two of these occasions the degree of bloat approached dangerous levels. Arbitrarily, when the pressure within the rumen reached a level of 50 mm. Hg above atmospheric pressure, treatment was initiated. This consisted of the intrarumen administration (via stomach tube) of

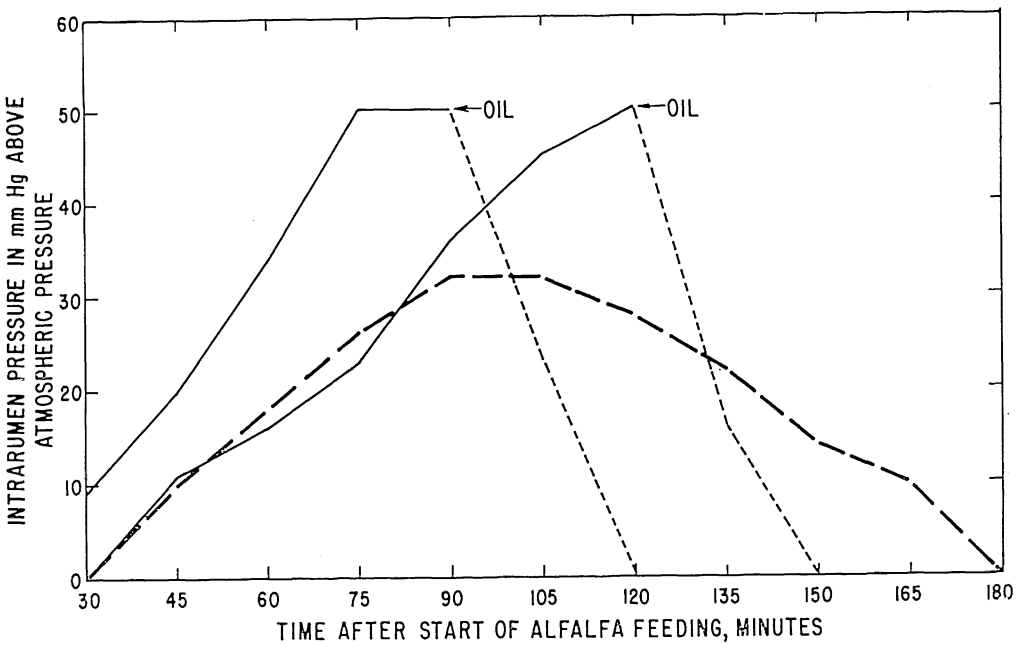


FIG. 1. Average changes of intrarumen pressure with time after feeding fresh alfalfa for Cow 54 on four occasions without treatment, as compared with the changes of pressure on two occasions when bloat was treated by the intrarumen administration of vegetable oil.

100 ml. of vegetable oil (Wesson). Figure 1 presents the average intrarumen pressures of Cow 54 at 15-min. intervals following the start of the feeding period for the four occasions when oil was not given, as well as the pressure changes before and after oil administration on the two occasions when treatment was necessary. During the latter occasions, there was a rapid reduction of pressure resulting from large eructations which followed the administration of oil. This rapid fall of intrarumen pressure is in marked contrast to that associated with the spontaneous recovery from bloat, as shown on the four occasions when oil was not given. These observations are similar to those reported by Johns *(7)* in cattle fed red clover, and indicate that the formation of a stable intrarumen foam was responsible for the bloat. The average rise and fall of intrarumen pressure following the feeding of fresh alfalfa for Cow 54 (as depicted in Figure 1) was typical of that observed in the other experimental animals.

Influence of fresh vs. dehydrated alfalfa on ruminal motility and eructation. The average levels of resting intrarumen gas pressure, rate of ruminal contractions, interval between eructations, and the amount of gas eructated for Cow 897 during consecutive 20-min. periods after feeding fresh as compared with dehydrated alfalfa are presented (Figure 2). Each curve represents the average values obtained in three trials. Bloat did not occur in any of the trials, the intrarumen gas pressure remaining considerably below atmospheric pres-

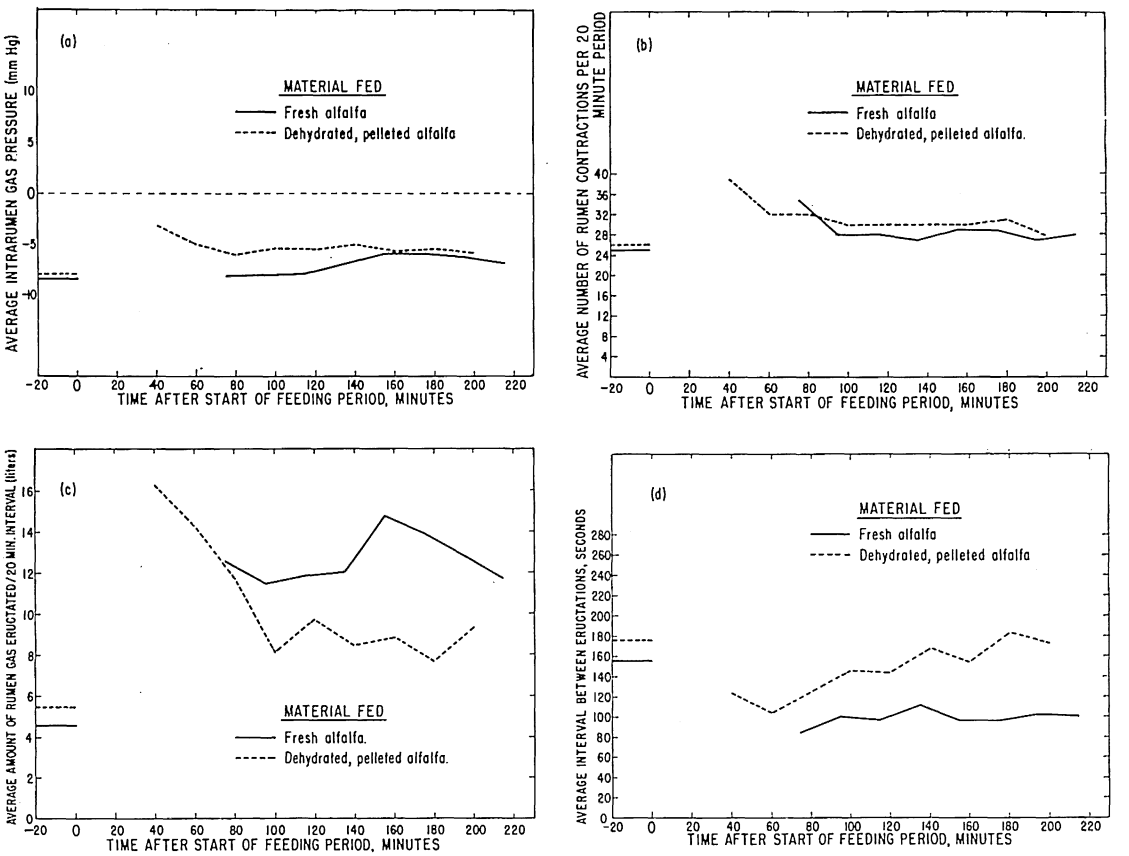


FIG. 2. Average variations of intrarumen pressure, eructation, and ruminal motility of Cow 897, after feeding fresh as compared with dehydrated, pelleted alfalfa.

sure (Figure 2a). Likewise, the rates of ruminal contractions were nearly the same in all trials (Figure 2b). The amount of gas expelled through eructation (Figure 2c) and, thus, the ruminal gas production, since the intrarumen gas pressure remained constant (Figure 2a), was greater on fresh as compared with dehydrated alfalfa for the period from 90 to 200 min. after feeding. Whether or not total gas production during the entire period of the trials was greater on the fresh alfalfa can not be determined from the data, since the gas eructated during the first hour after feeding fresh alfalfa and during the first ½ hr. after feeding dehydrated alfalfa could not be collected. However, it would appear either that more gas is produced per unit of dry matter from fresh alfalfa during the period of the trials, or that there is a considerable lag in gas production on fresh in comparison with dehydrated alfalfa. The former would seem to be more correct in view of previous reports, which have demonstrated maximal gas production during the first 90 min. after feeding fresh alfalfa *(3)*. In contrast to the steady decrease of eructation rate on dehy-

drated alfalfa, the interval between eructations in the fresh alfalfa trials remains quite constant (Figure 2d). This is responsible for the low intrarumen pressures observed in the latter, which occur in spite of the relatively high level of gas production.

DISCUSSION

Results of this investigation demonstrate that, at the levels fed, commercial dehydration and pelleting reduce the bloat-producing ability of fresh alfalfa. Previous work has indicated that other methods of dehydration may have a similar effect. Thus, Mead, Cole, and Regan *(10)* observed that alfalfa tops dried in a fruit dehydrator produced only two slight cases of bloat when fed to three cows over a four-day period. Control data were not reported. Johns *(7)* found a slight inverse relationship between the severity of bloat and the dry matter content of wilted, red clover. However, some animals developed moderate to severe bloat when fed sun-cured red clover hay. Sun-curing may have different effects on plant constituents than dehydration and these results may not be comparable to those reported in this paper. However, we have observed mild bloat in cows fed relatively large amounts of dehydrated alfalfa meal *(1)*.

The factors responsible for reducing the capacity of dehydrated alfalfa to produce bloat are not evident from the data presented. Since bloat did not occur on the dehydrated material, either the amount of gas produced per unit of dry matter was less and/or the efficiency of eructation was greater than on the fresh alfalfa. The measurements of ruminal gas production and eructation using Cow 897 seem to indicate that less gas was produced per unit of dry matter from the dehydrated alfalfa. However, as mentioned previously, this may not be true, because the gas produced and eructated during the first hour after feeding fresh alfalfa was not collected. A comparison of the eructation efficiency of Cow 897 after receiving the two types of alfalfa does not reveal any differences, because this particular animal did not bloat on either material, possibly because of the relatively small amount of alfalfa consumed.

If a reduction of ruminal gas production is not the complete explanation for the lack of bloat associated with dehydrated alfalfa, eructation must have been more efficient, probably because a stable foam did not develop within the rumen. Most investigators agree that the formation of a stable intrarumen foam which interferes with eructation is responsible for bloat on fresh legumes *(5)*. The question is, how does dehydration interfere with the formation of this foam? Clark and Weiss *(2)* and Weiss *(12)* have reported that the formation of a stable foam from fresh legumes depends upon an increase of the consistency of ruminal ingesta resulting from a lack of coarse material, which normally initiates salivary secretion by stimulating receptors in the mucous membranes of the rumen. Hungate *et al. (6)* have postulated that specific slime-producing bacteria within the rumen are responsible for the production of foam. Quin *(11)* and Lindahl *et al. (9)* have suggested that plant saponins may contribute

to the formation of a stable intrarumen foam. In the investigation reported in this paper, it would seem that these factors do not explain the marked difference in the severity of bloat attained on fresh as compared with dehydrated alfalfa. The latter was ground before pelleting and should be no more effective than the fresh material in stimulating the rumen to initiate a reflex secretion of saliva, although it is possible that the dry nature of the dehydrated alfalfa might cause a considerable production of saliva. Presumably, dehydration does not change the saponin content of the alfalfa or its ability to induce the formation of a specific type of rumen microorganism. Another constituent of legumes which may be responsible for the production of foam, and thus play an important role in the etiology of bloat, is water-soluble plant protein. In a previous investigation *(1)*, it was found that bloat could be produced in cattle fed dehydrated alfalfa by drenching with soluble protein (fresh eggwhite). The eggwhite resulted in the formation of a stable foam which interfered with eructation. It is conceivable that dehydration may reduce the bloat-producing ability of alfalfa by causing a denaturation of soluble protein.

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EFFECT OF CERTAIN ACUTE STRESS CONDITIONS ON THE PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS AND PROTEIN-BOUND IODINE IN DAIRY CATTLE [1]

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SUMMARY

Levels of plasma 17-hydroxycorticosteroids and protein-bound iodine were determined in 21 dairy cows suffering from various acute stress conditions, including surgery, acute mastitis, milk fever (parturient paresis), uterine prolapse, retained placenta, and metritis. The mean level of plasma 17-hydroxycorticosteroids in the animals under stress (11.95 μg. %) was elevated markedly over that of 20 normal cows (mean of 4.58 μg. %); whereas, the level of plasma protein-bound iodine in the cows under stress (3.63 μg. %) was not significantly lower than that of the control cows (4.22 μg. %).

It is established that various stressful conditions will cause elevated levels of plasma 17-hydroxycorticosteroids. Franksson and Gemzell *(6)* and Franksson *et al. (7)* have shown that preoperative anxiety as well as surgery in man caused elevated levels of plasma 17-hydroxycorticosteroids. Sandberg *et al. (15)* found that human patients postoperatively failed to metabolize hydrocortisone as rapidly as preoperatively, and that this may have contributed to elevated levels of 17-hydroxycorticosteroids following anesthesia, during and after surgery. Sandberg *et al. (14)* reported that dying human patients cleared intravenous hydrocortisone from the plasma at reduced rates and thus contributed to the rise in plasma levels of the corticoids in these patients. Tyler *et al. (17)* found that impaired liver function as shown by bromsulphalein was directly related to postoperative rises in plasma 17-hydroxycorticosteroids in man. Robertson *et al. (11)* have reported that the disease of ketosis in dairy cattle is associated with elevated levels of plasma 17-hydroxycorticosteroids and lowered levels of plasma protein-bound iodine, and that such animals also exhibited impaired liver function as measured by bromsulphalein fractional clearance *(13)*.

Recent work suggests that stressful agents and adrenocortical hormones have a depressing effect upon thyroidal activity. Williams *et al. (18)* have shown that adrenaline, typhoid vaccine, and trauma depressed the rate of I^{131} uptake by the thyroid, and Paschkis *et al. (10)* and Bogoroch and Timiras *(2)* have shown that formalin injections have a similar action also in rats. Fasting has

Received for publication October 14, 1957.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Dairy Science, New Brunswick. This work was supported in part by a grant from Merck Sharp & Dohme Research Laboratories, Division of Merck & Company, Rahway, New Jersey.

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been shown by Bondy and Hagewood *(3)* to decrease protein-bound iodine in rats and by Mixner *et al. (9)* in dairy bull calves; whereas, Blincoe and Brody *(1)* report that starvation of cows caused a marked decline in thyroid uptake of I^{131}. Engstrom and Markardt *(5)* reported that uncomplicated surgical stress or acute medical illness in man was not associated with abnormal serum-precipitable iodine, whereas chronic illness and hypoalbuminemia were associated with low serum-precipitable iodine. Sherer and Siefring *(16)* have reported that prednisone and prednisolone in humans depressed serum protein-bound iodine, thyroidal 24-hour I^{131} uptake, and the rate of secretion of hormonally bound I^{131}.

This study reports the effects of a variety of acute stressful conditions in dairy cattle on plasma levels of 17-hydroxycorticosteroids and protein-bound iodine.

METHODS AND PROCEDURES

The 21 cows under stress in this study were located in private dairy herds which were under the veterinary supervision of one of the authors (W.W.B.), who made the diagnosis in each case. These animals were acutely ill (all chronic illnesses were excluded) and were animals which had calved within 6 wk. The 20 normal animals in this series also had calved within 6 wk. Both groups were composed of animals of the Holstein and Guernsey breeds.

Blood glucose and acetone were determined on all blood samples and in all instances were found to be in the normal range. Plasma 17-hydroxycorticosteroids (17-OH-CS) were determined by the method of Robertson and Mixner *(12)* and plasma protein-bound iodine (PBI) by the method of Brown *et al. (4)* as modified by Lennon and Mixner *(8)*.

RESULTS AND DISCUSSION

The data are summarized (Table 1). The 21 animals under stress had a mean 17-OH-CS level of 11.95 μg. %, which was significantly higher than the mean of the normal level (20 cows) of 4.58 μg. % ($P<0.01$). The mean plasma level of PBI in the animals under stress (3.63 μg. %) was lower than the mean of the normal animals (4.22 μg. %), but the difference was not statistically significant ($P>0.05$).

These data clearly indicate that there are a variety of acute stressful conditions in dairy cattle which are associated with marked increases in plasma levels of 17-OH-CS and at the same time having very little, if any, effect on the level of PBI. It is not known to what extent actual adrenocortical stimulation and possible depression in rate of peripheral utilization of the endogenous corticoids interacted to cause the elevated levels of 17-OH-CS in the animals under stress. However, the stressful situations were known to be acute and had existed for only short periods of time. This may have been the reason that mean PBI level was not markedly depressed in the animals under stress, as might have been expected if the stresses had been chronic ones *(5)*.

TABLE 1

Plasma levels of 17-hydroxycorticosteroid and protein-bound iodine in normal dairy cows and cows under stress

Animal group and cow number	Plasma 17-OH-CS	Plasma PBI	Diagnosis or remarks
	(μg. %)		
Normal cows	4.58	4.22	Values are the means for 20 normal cows
Cows under stress			
1	12.34	4.96	Acute mastitis, temp. 105.6° F.
2	15.55	4.27	Acute mastitis, scours, temp. 105° F.
3	9.47	4.31	Hardware, mastitis, temp. 106° F.
4	11.14	2.83	Acute mastitis, temp. 106° F.
5	17.24	1.60	Acute mastitis
6	10.63	4.04	Acute mastitis, temp. 104.6° F.
7	8.75	4.66	Milk fever (parturient paresis)
8	8.56	2.14	Milk fever (parturient paresis)
9	9.41	2.34	Milk fever (parturient paresis)
10	18.29	3.91	Retained placenta, nephritis, metritis
11	12.39	2.55	Retained placenta, pyelonephritis
12	6.58	1.98	Retained placenta, metritis, mastitis, temp. 104° F.
13	6.60	3.53	Retained placenta, metritis
14	7.86	1.65	Retained placenta
15	14.21	8.92	Metritis, toxemia
16	13.09	2.01	Complete uterine prolapse
17	13.58	4.42	Rumenotomy, traumatic gastritis
18	17.84	9.19	Rumenotomy, traumatic gastritis
19	7.01	2.38	Rumenotomy, traumatic gastritis
20	8.67	5.36	Hardware in rumen
21	21.78	2.87	Listerelosis
Means of 21 cows under stress	11.95	3.63	

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PHENOLSULFONPHTHALEIN FRACTIONAL CLEARANCE IN DAIRY CATTLE AS A MEASURE OF RENAL FUNCTION[1]

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SUMMARY

A new test for renal function in dairy cattle has been devised, based on the rate of clearance of phenolsulfonphthalein (PSP or phenol red) from the blood and body fluids by the kidneys. Two bull calves and two lactating cows were used in these experiments, in which PSP was injected intravenously. The blood plasma level of PSP was determined at various time intervals after the injection. The plasma level of PSP fell rapidly at first and less rapidly thereafter. The over-all curve of plasma levels was differentiated into two first-order regressions in which the $\log_e$ of PSP concentration were inversely and rectilinearly related to the time after PSP injection. The first regression was theorized to represent the rate at which PSP moved from the plasma into other body fluids (collectively, its volume of distribution), with a mean slope value in the four animals of 0.171 per min. and a mean biological half-time of 4.4 min. The second regression was theorized to represent the rate at which PSP was eliminated from its volume of distribution (mean of 36.9% of body weight) by the kidneys and has been termed PSP fractional clearance. It is defined as that fraction of the total volume of distribution of PSP which is cleared of the dye per minute on an instantaneous basis. The mean PSP fractional clearance for the four animals was 0.0298, with a mean biological half-time of 24.5 min. PSP fractional clearance is easily measured and is considered to be a dynamic measure of renal function.

The use of phenolsulfonphthalein (PSP or phenol red) in a test for renal function in humans was introduced by Geraghty and Rowntree *(4)* in 1911, in which PSP was injected intramuscularly and the quantity of PSP excreted into the urine during the first and second hours was used as the criterion of renal function. A person having normal renal function will eliminate 40 to 50% of PSP during the first hour and 60 to 75% of the total within 2 hr. That PSP is eliminated by glomerular filtration and by tubular secretion was proven subsequently in experiments by Sheenan *(16)*, Marshall *(8)*, Marshall and Grafflin *(9)*, and Goldring *et al. (5)*.

Plasma clearance techniques have been applied to PSP *(17)*, and showed that both dye and protein concentration in the plasma affected clearance values of PSP. Such values must also be corrected for differences in body surface area, to obtain comparable data.

In studies on hepatic function in the human *(6, 7)*, horse *(3)*, and cow *(2, 12, 13, 15)*, the technique of fractional clearance has been used with the dye bromsulphalein. Fractional clearance is independent in its expression of dye concentration and plasma volume or body weight, being essentially the slope

Received for publication October 11, 1957.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Industry, New Brunswick. This work was supported in part by a grant from Merck Sharpe & Dohme Research Laboratories, Division of Merck & Company, Rahway, New Jersey.

value of an exponential or first-order regression ($\log_e Y$ *vs.* X), which characterizes the rate of elimination of the substance from the blood, or volume of distribution.

Indications were evident that PSP might be eliminated from the blood and body fluids by the kidneys in a similar manner, and that the use of PSP fractional clearance as a measure of renal function would eliminate the need for collecting urine samples. This would greatly facilitate studies on renal function in farm animals, from which it is difficult to obtain quantitative urine collections, especially from the males.

Accordingly, studies were initiated to determine if the technique of fractional clearance with PSP might be used for the study of renal function in dairy cattle and, if so, to develop the quantitative aspects of the problem into a usable method which might have general application. A brief report has been made on this work *(10)*.

MATERIALS AND METHODS

Reagents for PSP assay:

1. Phenolsulfonphthalein (Hynson, Wescott and Dunning, Inc., Baltimore, Maryland) is prepared for infusion (6 mg. per ml.): 6 g. phenolsulfonphthalein, 9 g. NaCl, and 17 ml. *N* NaOH, made up to one liter with sterile, double distilled H_2O.
2. 0.9% NaCl solution
3. 0.5 *N* NaOH solution
4. 0.17 *N* HCl solution
5. Anti-coagulant, 24 g. ammonium oxalate, 16 g. potassium oxalate per liter of water; 1 ml. per 10 ml. of blood.

Quantitative determination of PSP in plasma:

1. A control plasma sample from a given cow and a PSP plasma sample from the same cow were each divided into two separate 2.0-ml. portions in colorimeter tubes. To one portion of each plasma sample 3.0 ml. of 0.17 *N* HCl was added, and to the other portion 3.0 ml. of 0.5 *N* NaOH, to develop the dye color. Each were mixed thoroughly and let stand 10 min.
2. For each plasma sample in turn, the spectrophotometer (560 mμ) or colorimeter was zeroed on the corresponding acid blank. Then the optical density of the corresponding alkali-developed tube was read. A Beckman Model B Spectrophotometer was used in these studies.
3. The optical density (O.D.) of the control plasma tube was subtracted from the O.D. of the PSP plasma tube, to obtain an adjusted optical density (A.O.D.) for the plasma unknown tube.
4. A standard PSP tube was run in the same way, in which PSP was added both to a control plasma acid tube and to a control alkali tube.
5. Calculation of concentration of PSP:

$$\text{PSP, mg. per ml.} = \frac{\text{mg. of standard PSP} \times \text{A.O.D. of unknown plasma} \times 0.5}{\text{A.O.D. of standard PSP}}$$

EXPERIMENTAL PROCEDURE

Two Holstein bull calves and two lactating Holstein cows were injected rapidly in the jugular vein with varying quantities of PSP. Small blood samples were collected initially and at 2-min. intervals from 3 to 15 min. after injection and at 15-min. intervals from 30 to 120 min. after injection. The time was noted accurately at the middle of the injection and of each blood collection period, to establish accurate time relationships. PSP concentrations were determined on all plasma samples by the method described.

RESULTS AND DISCUSSION

The plasma levels of PSP in the four animals declined in an essentially identical manner with time following injection, and are presented graphically (Figure 1) for Bull Calf H711[7] (body wt., 46.3 kg.), which was injected with

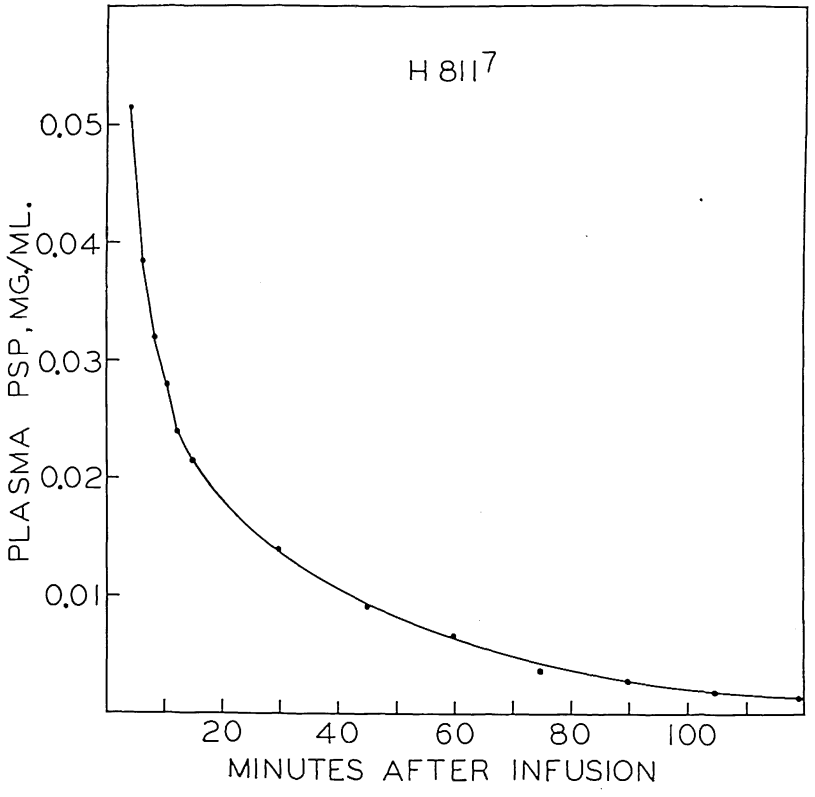


FIG. 1. Decline in plasma level of phenolsulfonphthalein (PSP) in Bull Calf H811[7], with time after infusion, showing the rapid initial decline and a more gradual decline thereafter.

480 mg. of PSP. The level of plasma PSP declined rapidly during the first 15 min. and less rapidly thereafter. An analysis of this curve, according to the methods outlined by O'Neal *(14)* for thyroxine, showed that this was actually a composite curve consisting of two components, each of which may be expressed

as a first-order regression with an exponential rate of decline (Figure 2). It is postulated that the initial rapid decline in PSP concentration of the plasma, as represented by the initial first-order regression, is owing to the distribution of PSP from the plasma into certain of the body fluids and that the slower decline in PSP concentration, as shown by the second first-order regression, is due to the elimination of PSP by the kidneys after distribution has been completed.

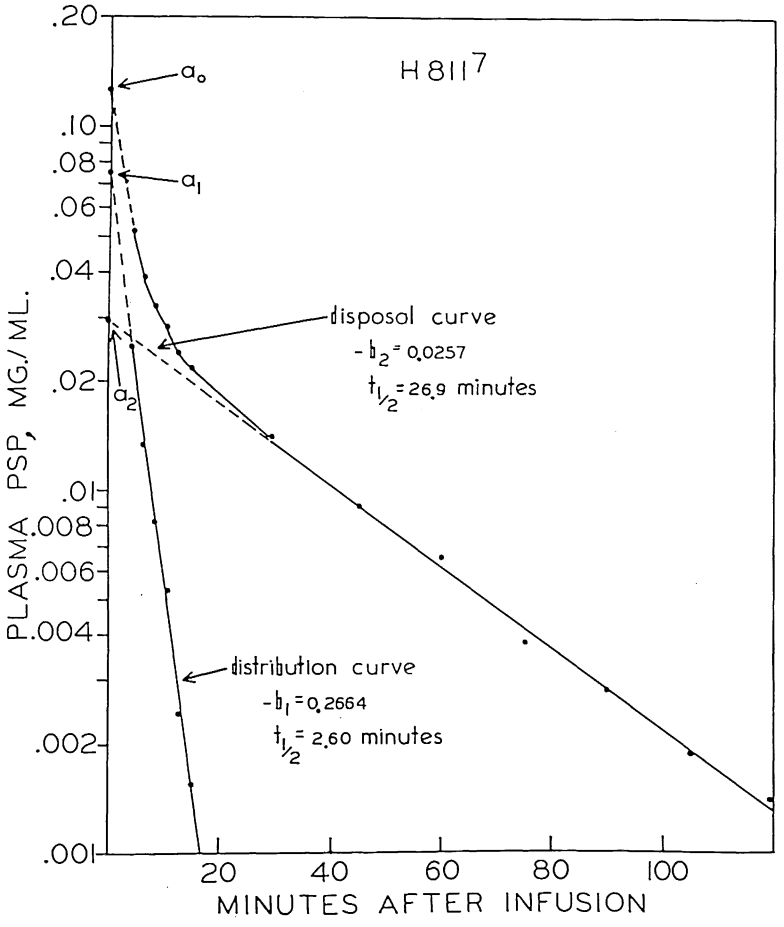


FIG. 2. Curve shown in Figure 1 is actually a composite curve, consisting of two first-order regressions as shown in this figure. The distribution curve (half-life of 2.60 min.) represents the rate at which PSP is moving into its volume of distribution. The disposal curve represents the rate at which PSP is being eliminated from the body by the kidneys. Its slope or $-b_2$ value (0.0257) is termed PSP fractional clearance and is the direct measure of renal function (Bull Calf H811[7]).

Thus, the scheme for the disappearance of PSP from plasma may be represented by the general formula

$$\text{PSP, mg. per ml.} = a_1 e^{-b_1 t} + a_2 e^{-b_2 t}$$

in which a_1 = concentration of PSP (mg. per ml. of plasma) at zero time by extrapolation of the more rapid initial distribution curve

$-b_1$ = slope (rate constant) of distribution curve

a_2 = concentration of PSP (mg. per ml. of plasma) at zero time by extrapolation of the slower disposal curve

$-b_2$ = slope (rate constant) of the slower disposal curve

e = base of natural logarithms

t = time in minutes from dye injection

It is apparent from this scheme that the volume of distribution (V.D.) of PSP may be calculated from the following formula:

$$\text{V. D., ml.} = \frac{\text{mg. of PSP injected}}{a_2}$$

The biological half-time or -life ($t_{1/2}$) of either of the rate reactions may be calculated by dividing 0.693, the natural logarithm of 2, either by b_1 or by b_2.

The rate constant ($-b_2$) of the disposal curve is the direct measure of kidney function and will hereafter be referred to as "PSP fractional clearance." This is the actual fractional decline in the level of PSP in the plasma per minute on an instantaneous basis, and may be defined as the fractional part of the total volume of distribution of PSP which is cleared of the dye per minute, also on an instantaneous basis.

Table 1 presents a summary of the kinetic characteristics of the distribution and disposal of PSP in the four animals used. The mean biological half-time ($t_{1/2}$) of distribution of the dye, of 4.42 min., indicated that the distribution phase for the dye would be about 99%, completed in 30 min. (seven half-lives). The mean PSP fractional clearance ($-b_2 = 0.0298$) gives a mean biological half-time ($t_{1/2}$) of 24.4 min. Since two points will define a first-order regression curve plotted on a semilogarithmic basis, a routine determination of PSP fractional clearance could be based on the collection of two blood samples after the injection of the dye. From the above considerations, it is evident that the first sample should not be taken sooner than 30 min. after dye injection, to allow the dye distribution phase to be completed. The second sample might then be conveniently taken after a second 30-min. period, which would be longer than the mean half-life for the disposal phase of 24.4 min. as measured on the four animals.

The following formula would be used to calculate PSP fractional clearance:

$$\text{PSP fractional clearance } (-b_2) = \frac{2.3\,(\log_{10}\text{A.O.D.}_{t_1} - \log_{10}\text{A.O.D.}_{t_2})}{t_2 - t_1}$$

where A.O.D._t is the adjusted optical density of the first plasma sample taken at 30 min. after dye injection (t_1), and A.O.D._t, the corresponding value for the second plasma sample taken at approximately 60 min. after dye injection (t_2).

TABLE 1

Kinetic characteristics of the distribution and disposal of phenolsulfonphthalein (PSP) in dairy animals

Characteristic	Bull calves		Lactating cows		Means
	HS11[7]	HS90[7]	H661	H804	
Body wt., kg.	46.3	36.4	740.3	711.2	
Dosage PSP, mg.	480	240	1128	1140	
Distribution curve					
degrees of freedom	5	6	6	6	
r[b]	−0.995**	−0.990**	−0.996**	−0.996**	−0.994**
a_1, mg/ml	0.0750	0.0330	0.0198	0.0239	
$-b_1$, per minute	0.266	0.124	0.138	0.155	0.171
$t_{1/2}$, minutes	2.60	5.60	5.02	4.46	4.42
Disposal curve					
degrees of freedom	6	6	5	4	
r[b]	−0.998**	−0.985**	−0.960**	−0.986**	−0.982**
a_2, mg/ml	0.0295	0.0148	0.0034	0.0106	
$-b_2$, per minute[a]	0.0254	0.0224	0.0300	0.0413	0.0298
$t_{1/2}$, minutes	27.3	30.9	23.1	16.8	24.5
Volume of distribution					
ml.	16,300	16,200	331,700	107,700	
% of body wt.	35.2	44.5	44.8	15.1	36.9

** Significant at the 1% level ($P < 0.01$).

[a] This is the "PSP fractional clearance," measure of renal function.

[b] The coefficient of correlation between time in minutes (X) after dye injection and $\log_e$ of PSP concentration (Y) of the first-order regressions.

If more than two plasma samples are collected during the dye-disposal phase, the method of least squares can be appropriately used to calculate the PSP fractional clearance, or $-b_2$ slope value. The natural logarithms of dye concentration per ml. would be used as the Y-variable and t_1, t_2, ----, t_n, in minutes as the X-variables.

Nineteen determinations of kidney function were run in which blood samples were collected at 30, 40, 50, and 60 min., to determine the correspondence between PSP fractional clearance values based on the two blood samples taken at 30 and 60 min. (calculated by the formula), and values based on all four blood samples taken at 30, 40, 50, and 60 min. after PSP injection (calculated by method of least squares). The mean PSP fractional clearance values were 0.0349 and 0.0348 as calculated by the two methods, respectively. An analysis of variance (37 total degrees of freedom) indicated that the difference between these means was not statistically significant. This would indicate that two blood samples taken at 30 and 60 min. following PSP injection would be sufficient in a routine test.

The recommended routine procedures to be followed in running PSP fractional clearances in addition to those already presented may be outlined as follows: Collect 15 ml. of blood from the animal before the infusion of the dye. Then rapidly inject 1.5 mg. of PSP per lb. of body weight into a jugular vein. Record the time at the midpoint of the injection (t_o). At approximately 30 and 60 min. from t_o, collect 15-ml. samples of blood, noting in each instance the

exact time when half of the blood sample has been collected. The elapsed time in minutes from t_0 to the middle of the 30-min. blood collection equals t_1, whereas t_2 equals the corresponding elapsed time for the 60-min. blood collection. Centrifuge the blood samples to prepare plasma.

Date collected on Bull Calf H811[7] (Table 1) are used to illustrate a typical calculation of PSP fractional clearance. The level of dye injected could have been reduced from 480 to 160 mg. and still have been sufficient according to the recommended dosage level.

Data:

Animal H811[7]; date of trial, 2-14-57; body wt., 46.3 kg. or 101.9 lb.; wt. of PSP injected, 480 mg.

Plasma tube	Elapsed time	Optical density	Adjusted optical density
	(min.)	*(O.D.)*	*(A.O.D.)*
Preliminary plasma[a]	$t_0 = 0$	0.0386	.....
30-min. plasma	$t_1 = 29.8$	0.3570	0.3184
60-min. plasma	$t_2 = 59.8$	0.1871	0.1485

[a] Subtract O.D. of preliminary plasma from 30- and 60-min. O.D. to obtain A.O.D.

Calculations:

$$\text{PSP fractional clearance } (-b_2) = \frac{2.303(\log_{10} 0.3184 - \log_{10} 0.1485)}{59.8 - 29.8} = 0.0254$$

The PSP fractional clearance test, as described, is a new and convenient test for renal function, which is performed on blood plasma. The concept of fractional clearance is a relatively new one, being applied first by Lavers *et al. (7)* with bromsulphalein, in a new liver function test. It seems to be admirably adapted to the study of kidney function as well, and because the measure is essentially an expression of the rate of a first-order reaction, it is independent in its expression of the amount of dye injected (within limits) and the size of the animal. Plasma clearance as used in the conventional sense would be related to the size or surface area of an animal and would have to be corrected accordingly; whereas, fractional clearance is not subject to this limitation.

The evidence from this work is that PSP has a much larger volume of distribution in the body than plasma volume, being about 36.9% of body weight (Table 1). This is identical to that reported for the thyroxine space in dogs *(14)*, similar to that found for the thyroxine space in hyperthyroid human subjects *(1)*, and similar to the L-triiodothyronine space in cattle *(11)*.

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TECHNICAL NOTES

DETERMINATION OF THE TOTAL NITROGEN IN STORED MILK BY NESSLERIZATION AND BY THE MACRO-KJELDAHL METHODS

The total nitrogen in milk can be determined by direct Nesslerization of the digested sample. Hetrick and Whitney *(5)* reported that this method for whole milk yielded results that averaged 2.5% lower than those obtained by the Kjeldahl-Gunning-Arnold method.

The direct Nesslerization method was used in this laboratory for determining the nitrogen distribution in sterilized milk held at different temperatures. The data showed unexplained variations in the total nitrogen of milk preheated to 140 and 180° F., sterilized at 305° F., homogenized and stored at 40, 70, and 100° F. Since there was no information in the literature to explain these variations, it was decided to compare the Nesslerization with the modified Macro-Kjeldahl method, which is the same as the official one *(1)* except for the following modifications: 5 ml. of milk was pipetted instead of weighed; 85 ml. of a mixture of sodium hydroxide and sodium thiosulfate solution (prepared by mixing 1.5 liters of 50% sodium hydroxide solution with 200 ml. of a solution containing 80 g. of sodium thiosulfate) were added before distillation; the ammonia was collected in a 4% boric acid solution containing 5 ml. of indicator per liter (a solution of 0.5 g. methyl red and 0.2 g. methylene blue in 500 ml. of 95% ethanol), and the distilled ammonia was titrated against standard acid to the original grey color of the indicator.

For comparing both methods, fresh, raw whole milk was preheated to 140° F., homogenized, sterilized at 305° ± 0.25° F., cooled to 45° F., collected in sterile cans and, finally, aseptically bottled and stored at 70° F. *(3)*. Samples were removed at intervals and analyzed for total nitrogen by both methods. The results are presented (Table 1).

The results show that the total nitrogen as determined by the modified Macro-Kjeldahl method gave consistent results, but the direct Nesslerization method gave varying results. Erdman *(2)* has observed that some of the nitrogen in certain organic materials is converted to amines instead of ammonia. The amines are titratable as ammonia in the Macro-Kjeldahl method, but would not develop the same color with Nessler's reagent. This was emphasized also by Hetrick and Whitney *(5)*. Also, Jamil *(4)* reported that the Nesslerization method yielded higher nitrogen values for certain foods than did the Macro-Kjeldahl method. In the present study, both low and high nitrogen values were obtained by the direct Nesslerization method. These variations might be explained on the basis of the observations made by Erdman *(2)* and Jamil *(4)*.

TABLE 1

Comparison of the direct Nesslerization and the modified Macro-Kjeldahl methods for determining total nitrogen in raw, freshly sterilized and sterilized milk stored at 70° F.

Treatment	Direct Nesslerization			Modified Macro-Kjeldahl		
	mg.N/100 ml. milk					
			Av.			*Av.*
Raw	540.4	541.2	540.8	540.0	538.0	539.0
Freshly sterilized	543.5	542.6	543.0	540.0	537.0	538.5
Sterilized milk stored at 70° F.:						
3 days	515.2	521.1	518.2	535.9	537.9	536.9
7 days	525.8	524.0	524.9	531.9	533.9	532.4
10 days	508.5	509.2	508.8	537.9	535.9	536.9
14 days	510.0	525.8	517.9	535.4	539.4	537.4
17 days	526.1	554.1	526.0	535.4	537.4	536.4
21 days	554.1	554.1	554.1	535.4	535.4	535.4
24 days	572.1	572.0	572.0	535.4	537.4	536.4
28 days	531.0	533.6	532.3	537.4	535.4	536.4

On the basis of the work carried out at this laboratory, it is indicated that the direct Nesslerization method is suitable for nitrogen determinations in fresh milk, but that it can not be used for the study of nitrogen distributions in sterilized milk stored at different temperatures.

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CENTRIFUGAL TUBE FOR EXTRACTING BUTYL ALCOHOL IN THE PHOSPHATASE TEST

Culture tubes, 1.6 cm. by 15 cm., are used to develop color in the phosphatase test of Sanders and Sager.[1] The color is extracted with 5 ml. of butyl alcohol and the sample is centrifuged with special tubes that fit into the trunnion cups. Four milliliters of the extracted butyl alcohol is used for the photometric estimation of phenol. If more than 4 ml. is needed, it is suggested that large tubes be used and the color extracted with the necessary amount of butyl alcohol in both the test and standard. However, it was found that culture tubes for centrifugation were not satisfactory.

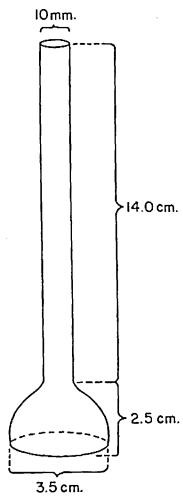


Fig. 1. Centrifugal tube for extracting butyl alcohol in the phosphatase test.

A modified Pyrex tube, which fits into the trunnion cup of a Babcock centrifuge 40.6 cm. in diameter, was designed. It is shown in Figure 1 with dimensions. The capacity of the bulb is 10-12 ml., which is about equal to the total volume of test solution and the reagents used to develop color. The capacity of the neck is 10-11 ml. and it is wide enough to admit a 10-ml. pipette, which is used to add the reagents and to withdraw the alcohol layer.

A number of these tubes were made by a glass-blower in the University. As yet they are not being made commercially.

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OZONE—CAUTION!

Within recent years ozone generators have been installed in cheese-curing rooms and in food-storage lockers in an effort to retard contamination by mold. A report appearing in a recent issue of the *Medical News Letter,* 30 (9), 1957, pronounces ozone to be a public health hazard. Although no human fatalities have been reported as a result of exposure to ozone, at concentrations in excess of 0.1 p.p.m. by volume definite symptomatic effects are evident, the most pronounced effects being pulmonary edema and hemorrhage.

The hazards involved are considered to outweigh the value of an ozonizer as a deodorizer or gemicidal agent in confined working areas. Where ozonizers are installed, warning signs should be posted and personnel cautioned not to occupy these lockers longer than is necessary.

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PRELIMINARY REPORT ON TESTS OF THE ACCURACY OF MILK SCALES

Measuring the milk production of individual cows by weighing the milk at the completion of milking has been practiced for many years. Production records are desirable for individual herd management and for herd improvement association records. Recently, during discussions on milk-flow meters, the question of accuracy was raised. It was evident that standards must be set up to provide a basis for approving meters or other weighing devices, for subsequent use in production-record programs. It was generally agreed that such standards be equal to, or preferably better than, existing standards.

It then became necessary to determine, as accurately as possible, the prevailing standard of accuracy of milk scales. It was obvious that a true determination of accuracy standards would have to be based on checks of scales throughout the major milksheds of the nation. Such a task would be formidable and virtually impossible to accomplish. It was then decided to check a few scales in a preliminary study, to obtain an indication of scale accuracy.

Test in Minnesota. Twenty milk scales in actual use by D.H.I.A. testers in the Minneapolis-St. Paul milkshed were checked with standard weights. The readings were made to the nearest 0.05 lb.

The weight data were obtained in 5-lb. increments from 5 to 50 lb., but were summarized at the 15,- 25-, 35-, and 45-lb. readings. Of the 80 readings, 22 provided a zero error, three provided a negative error, and 55 provided a small positive error. Only two scales provided readings of more than 1% error (0.2 lb. at 15 lb., or 1.33%). Three scales provided 1% error readings (two at 25 lb. and one at 35 lb.). Individual scale averages varied from 0.00 to 0.97% error, with an over-all average of 0.32% error.

Tests at Beltsville, Maryland. A typical spring milk scale was checked in the Dairy Cattle Research Branch barn during a ten-day test involving ten cows milked twice each day. Two hundred comparative weighings were made on the spring scale and on a precision bench scale.

The milk weights varied from 4.85 to 41.0 lb., averaging 17.6 lb. The spring scale and the bench scale agreed during 105 of the 200 readings. They differed by ± 0.05 lb. during 81 readings and by ± 0.10 or more pounds during 14 readings. Considering that the differences of ± 0.05 lb. could be due entirely to the readability of the scales, only 14 readings yielded a substantial difference. These 14 readings were all less than 1% in error, averaging 0.58%. In other words, 186, or 93%, of the readings were within ± 0.05 lb.; 194, or 97%, of the readings were within ± 0.10 lb. The over-all average error of the 200 readings was less than 1/25th of 1%.

An old, well-worn scale, which happened to be in use by a D.H.I.A. tester, was checked during these tests and found to be 1.8 to 2.0% in excess of the standard weights between 15 and 45 lb. This error was almost linear through the range and was probably due to spring fatigue, a common fault of older scales.

Check on new spring scales. A check of 11 new spring scales at Beltsville, with standard test weights at 15, 25, 35, and 45 lb., showed that all errors were less than 1%. The greatest percentage error was 0.60% and the over-all average error was 0.15%. Of the 44 readings, seven provided a zero error, seven provided a negative error, and 30 provided a positive error.

Summary

The average error of the D.H.I.A. milk scales which were checked varied from 0 to 1.89%, with an over-all average of 0.40%. Individual scale readings varied from 0 to 2% in error.

The weights obtained by milk scales for D.H.I.A. records, as checked in these tests, were found to have an average error of less than one-half of 1%.

New scales were found to have an error of less than one-sixth of 1%. The greatest error found in any weight reading was only 2%, and that was found on an old scale which should not have been in service.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. J. F. Kendrick, Acting Head, Dairy Herd Improvement Section, Dairy Cattle Research Branch, AHRD, USDA, Washington, D. C.; Mr. Ramer D. Leighton, Extension Dairyman, University of Minnesota and the Minnesota Agricultural Experiment Station, St. Paul, Minnesota.

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ISOLATION AND IDENTIFICATION OF λ-CASEIN

Waugh and von Hippel *(8)* have reported the presence of a casein component in cow's milk which they have named κ-casein, and they have characterized κ-casein in preparations which contained other casein components.[1] McMeekin, Groves, and Hipp *(3)*[2] have described the use of calcium chloride for the isolation of κ-casein from aqueous solutions of α-casein. A similar procedure, utilizing the difference in solubility exhibited by components of α-casein in the presence of calcium ions, has been employed in this laboratory *(4)* for the isolation of κ-casein. We have found that the κ-casein thus obtained is associated with a minor component which we have designated as λ-casein. λ-Casein has been isolated from the crude κ-casein preparation by differential centrifugation, and characterized with respect to *(a)* phosphorus content, *(b)* stability to calcium ions, *(c)* reaction with rennet, and *(d)* sedimentation behavior. A comparison of these properties with those of κ-casein *(7)* and α_R-casein (defined later), indicates that λ-casein was a separate and distinct protein component.

Preparation of the crude κ-casein fraction. Warner's *(7)* α-casein, for which the customary extraction with ether and alcohol has been omitted, was the starting material. Separation of α-casein into a calcium-soluble crude κ-casein fraction containing both κ- and λ-casein, and a calcium-precipitated α_R-casein fraction, was made by addition of calcium chloride to an unbuffered solution of α-casein at 5° C. and pH 6.6-7.0. Necessary adjustments in pH were made with 0.1 *N* NaOH and 0.1 *N* HCl. Details of the separation are:

a) A 2-3% α-casein solution was made 0.20 *M* with respect to $CaCl_2$ by the dropwise addition, with stirring, of 2.0 *M* $CaCl_2$. The solution was allowed to stand overnight. After this period, the majority of the calcium-sensitive α_R-casein had precipitated, whereas the supernatant was decanted and centrifuged as described, to remove the remaining α_R-casein.

b) The supernatant from *a*) was heated to 35° C. and centrifuged at 9,000 r.p.m. for 30 min. in a Servall Centrifuge.

c) The supernatant from *b*) was centrifuged for 60 min. at 25° C. in a Spinco Model L Centrifuge (No. 21 Rotor) at 21,000 r.p.m.

d) The supernatant from *c*) was dialyzed against distilled water to remove the calcium. This procedure was used because some protein was lost when the calcium ions were removed with oxalate.

e) The crude κ-casein was acid-precipitated and stored at −20° C. A yield of 17% of the original α-casein was obtained as crude κ-casein, and λ-casein comprised approximately 15-20% of the crude κ-casein fraction. Phosphate buffers used in the examination of these caseins were those employed by von Hippel and Waugh *(5)*. At pH 6.98, the ionic strength of the buffers was 0.1, and at pH 12.0 the ionic strength was 0.19. Phosphorus analyses were made by a method outlined by Allen *(1)* and Walker *(6)*.

The isolated crude κ-casein had a phosphorus content of 0.46%, and possessed the characteristics reported for κ-casein *(8)*. Partial centrifugal fractionation of κ- and λ-casein indicates that the phosphorus content of κ-casein itself is less than 0.33%.

Examination of the ultracentrifuge pattern obtained from Warner's α-casein, in phosphate buffer at pH 6.98, revealed a large skewed peak and a small, faster-moving component. This fast-sedimenting component was absent from the α_R-casein pattern, which indicated that it may have been the source of the κ-casein.

Electrophoretic examination of the crude κ-casein fraction, at pH 6.98 in phosphate buffer, revealed one symmetrical peak with a mobility slightly less than that of the original α-casein. Waugh and von Hippel *(8)* reported that β-casein was present in their preparation of κ-casein.

Two peaks were observed when a 0.6% solution of crude κ-casein, in phosphate buffer at pH 6.98, was subjected to ultracentrifugation. The faster-sedimenting component, containing approximately 80% of the total protein, presented a sedimentation constant identical to that reported for κ-casein *(8)*, $S_{20} = 13.18$ Svedbergs. The slower component, λ-casein, moved with a $S_{20} = 1.10$ Svedbergs. At pH 12.01, all protein in the crude κ-casein fraction was present in a single polydispersed peak, with a $S_{20} = 1.10$ Svedbergs.

Preparation of λ-casein. The crude κ-casein from step *e)* was prepared as a 1.0% solution in 0.2 *M* NaCl at pH 7.0. This solution was centrifuged at 40,000 r.p.m. in a Spinco Model L Centrifuge (No. 40 Rotor) at 5° C. for 5 hr. The upper half of the supernatant, containing λ-casein free of κ-casein, was removed, dialyzed against distilled water, and concentrated by pervaporation.

The phosphorus content of the λ-casein was 1.18%. Although the phosphorus content of α_R-casein was 1.16%, λ-casein (as shown in Table 1), differs from α_R-casein in its sedimentation constant and stability to calcium ions.

λ-Casein, prepared as a 1.0% solution in distilled water, at 25° C., was not precipitated by calcium ions. At calcium ion concentrations of 8 m*M*, the casein solution became turbid but, as the calcium level was increased, this

[1] κ-Casein resembles a Z-casein fraction of Linderstrom-Lang *(2)*, in stability to calcium ions.

[2] The published abstract of this paper contains no reference to the isolation of κ-casein. This work was presented orally at the 1[illegible] National Meeting of the American Chemical [illegible]ety, April, 1957.

TABLE 1
Comparison of certain characteristics of α_R-, κ-, and λ-casein

Component	P	Stability to calcium ions *(0.2M)*	Visible reaction with rennet	S_{20}[a] × 10^{13}
	(%)			
λ-casein	1.18	Yes	No	1.10
α_R-casein	1.16	No	No	4.55
κ-casein	< 0.33	Yes*(8)*[b]	Yes*(8)*[b]	13.18*(8)*[b]

[a] Spinco Model E Ultracentrifuge, 59,780 r.p.m., at 25° C. S_{20} values are without partial specific volume correction. 0.6% protein in pH 6.98 phosphate buffers.

[b] These characteristics reported for κ-casein are as it existed in a mixture of casein.

condition decreased until, at concentrations of 100 m*M* and above, the solution remained clear. Under these same conditions, α_R-casein began precipitating from solution when the calcium concentration reached 5 m*M*.

At concentrations of λ-casein from 0.3 to 1.0% in phosphate buffer at pH 6.98, only one component was observed by ultracentrifugation. The sedimentation constant for a 0.6% solution of λ-casein was $S_{20} = 1.10$ Svedbergs. α_R-Casein under similar conditions had a sedimentation constant of $S_{20} = 4.55$ Svedbergs.

There was no visible reaction between λ-casein, free of calcium, and rennet.

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SPERMATOCRIT[1] VALUES IN FACILITATING THE ESTIMATION OF SPERMATOZOA CONCENTRATIONS

Investigation on the use of a special centrifuge for estimating spermatozoa concentrations in bull semen has given promising results. In this investigation, the centrifuge was used as directed by the manufacturer, except that the material centrifuged was undiluted semen instead of blood.

This method involves partially filling the capillary tubes with freshly collected semen. The small bore in these tubes facilitates filling, as the semen enters partly by capillary action. A tube can be partially filled easily, by holding it against the inside walls of the collection vials and slanting it just enough to allow the semen to enter. It is essential to leave about three-quarters of an inch of the tube unfilled, to enable heat-sealing without burning the semen.

The centrifuge used is especially designed to spin the tubes horizontally at 10,000 r.p.m. It has been determined that 10 min. of centrifuging will separate the spermatozoa from the seminal fluid and pack them firmly in the sealed end of the tube.

To express the packed spermatozoa as a percentage, a special graph supplied with the centrifuge is used to accommodate any volume sampled. The capillary tube is held against this graph in the position that puts the outside lines on the extremities of the column of semen in the tube. It is then necessary to count the number of lines covered by the column of packed spermatozoa to obtain the percentage of packed cells, which is equal to the number of lines counted. A small 12 × magnification lens helps considerably in making accurate counts of the lines.

Values of ½% to 18% have been obtained on the semen used in this study.

Duplicate measurements using this method were taken on all semen collected at the Central Experimental Farm, Ottawa, over several months in 1957. All samples were subjected to hemacytometer counts, to determine the association between the percentage of packed spermatozoa per cubic centimeter of semen. Results indicated that the percentage values were the logical independent variable, as the repeatability of duplicate percentages on the same

[1] Analogous to the usage of "hematocrit." "Hemato" is derived from the Greek and refers to blood; and "crit" originally spelled "krite," meaning to judge.

semen was 0.99; whereas, that for the hemacytometer counts was only 0.64.

Unfortunately, we were not able to use the popular photoelectric method of evaluating sperm concentrations concurrently. However, it is obvious that the photoelectric method, or any other method, would not be any more accurate than the centrifuge method, from the standpoint of repeatability.

From a practical standpoint, there are several distinct advantages for the centrifuge method over the photoelectric method. With the photoelectric method, a calibration of each instrument is necessary, and there is no assurance of the absence of unknown variations in electrical power or other factors affecting light transmission to the photoelectric cell. The centrifuge method is more direct, the only calibration needed being a measurement of the number of spermatozoa per cubic centimeter for each percentage of packed spermatozoa. This has been obtained by estimating the regression equation $(y = bx)$ of hemacytometer counts (y) on the percentage (x), and was found to be $y = (219.88 \pm 10.33)x$. Thus, by multiplying the percentages by 220, the estimated number of spermatozoa in millions per cubic centimeter of semen will be obtained.

The centrifuge method is easier to perform, also, as there is no glassware to be kept clean, as is the case with the photoelectric method. Too, the centrifuge method offers more flexibility in operation, because the semen can be sampled in the capillary tubes, sealed, and set aside for centrifuging after initial processing of the sampled semen. In the operation of the Experimental Farm's frozen semen project, the tubes are attached to the vials containing semen and, when the semen arrives at the freezing laboratory the capillary tubes are centrifuged, to estimate the spermatozoa concentration in the ejaculate collected several hours previously.

One precaution has to be observed in obtaining the percentage values. If many tubes are being centrifuged at one time, it is important to read all the percentages as soon as possible after the centrifuge stops. Otherwise, the spermatozoa will start mixing with the seminal fluid and there will be indistinct separation of the packed spermatozoa and seminal fluid.

It is suggested that the method just described be referred to as the Spermatocrit Method for determining spermatozoa concentrations.

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LACTOSE SYMPOSIUM[1]

PHYSICAL AND CHEMICAL ASPECTS OF LACTOSE

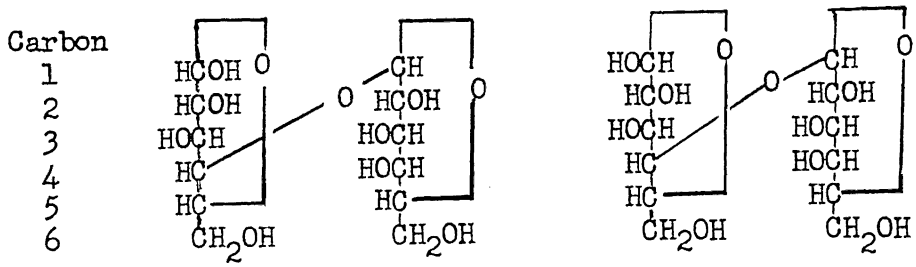
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The physical and chemical aspects of lactose is a broad subject to develop comprehensively during the time permitted in this symposium. Much of the information concerning the structure of lactose, the different forms and their kinetics and equilibria, was developed many years ago. An excellent review on lactose and its utilization has been published by Whittier *(9)* and, in addition, most textbooks on the chemistry of dairy products contain a chapter on lactose. Therefore, no attempt will be made in this paper to present a comprehensive review of the subject but only to emphasize the more important principles.

Lactose is a major constituent of milk and some of the by-products derived from milk. In round figures, it constitutes in percentages about 4.9 of milk, 5.1 of separated milk, 10 of evaporated milk, 38 of dry whole milk, 51 of nonfat dry milk, and 70 of dry whey solids. Unlike the two other major constituents of milk, namely, the milk proteins and butterfat, lactose is a relatively simple molecule, of which the structure has been elucidated. Furthermore, both alpha-lactose, in the form of the monohydrate, and beta-lactose, are commercially available in high purity.

Chemically, lactose is a disaccharide composed of a molecule of D-glucose and one molecule of D-galactose. The structural formula is shown (Figure 1). Before proceeding further, it may be of interest to review briefly the use of the terms D and L in sugar chemistry. In organic chemistry, the symbols D and L have sometimes been used to denote the direction of optical rotation of a compound, dextro, or D, being to the right, levo, or L, to the left. In carbohydrate chemistry, the terms refer to the configuration of the molecule, rather than to its optical rotation. The D series of sugar is the one in which the –OH group on the asymmetric carbon-5 is on the right, or on the same side of the lactal ring as the formula is written in the figure. The L sugars are the ones in which the –OH group on carbon-5 is on the left. Optical rotation is now indicated by the (+) or (—) signs.

[1] The four papers in this symposium were presented at the 52nd Annual Meeting of the American Dairy Science Association at Oklahoma State University, Stillwater. This is another example of a timely program, planned by an alert committee. The speakers are commended for their fine presentations. *Editor-in-Chief.*



Alpha Lactose Beta Lactose

FIG. 1. Structural formula—alpha- and beta-lactose

It will be noted from the structural formula that D-glucose and D-galactose each contains a 1-5 lactal ring, or a pyranose structure. These two molecules are connected through the aldehyde or, more correctly, the hemi-acetal group (C-1) of D-galactose and carbon-4 of the D-glucose molecule. Further, as a consequence of the hemi-acetal structure of monosaccharides, carbon-1 is asymmetric, which creates two isomers. The alpha D-sugar is the one which has the higher dextro-rotation and the –OH group is assigned to the right as the formula is written. The beta sugar is the one having the lower dextro-rotation, with the –OH to the left. In the case of lactose, it has been found that β-D-galactose is the component. However, in the glucose part of the molecule, the hemi-acetal group is not combined; therefore, it is possible to have both an alpha and a beta isomer. This accounts for the two forms of lactose which will be discussed in more detail later. For the moment, it is sufficient to note that lactose is 4-D-glucopyrano-syl-β-D-galactopyranoside. The actual proof structure is of interest.

As evidenced from the structural formula, lactose contains several types of functional groups. Since few of the chemical derivatives are of any practical importance, they will be mentioned only briefly. First of all, lactose possesses a glycosidic linkage which can be hydrolyzed by dilute acid or by the enzyme lactase to give glucose and galactose. Secondly, lactose contains a hemi-acetal group which participates in many interesting reactions, including those with phenylhydrazine, hydrocyanic acid, ammonia, hydroxylamine, etc., to form various condensation products. It is oxidized by bromine to lactobionic acid. However, like glucose, it does not react with such typical aldehyde reagents as Schiff's reagent and sodium bisulfite. In alkaline solution, it is oxidized by Fehling's solution, normally used for the quantitative determination of lactose. The reaction with Fehling's solution, however, is not stoichiometric.

In addition, the hemi-acetal group is believed to be important in the so-called browning reaction which sometimes occur in dairy products. In dry milk products under the influence of high moisture content, the initial step is believed to involve a condensation type of reaction between the hemi-acetal group and the amino group of amino acids or proteins, particularly the Σ-amino group of lysine. This initial complex then undergoes a series of as-yet-undefined intermediate reactions, resulting in the production of brown discoloration and off-flavor compounds.

Finally, lactose contains both primary and secondary alcohol groups which, to a large extent, account for its great solubility in water and lack of solubility in organic solvents. These alcohol groups are methylated by dimethyl sulfate or methyl iodide and silver oxide. The reaction has been useful in the proof of structure of lactose and other carbohydrates. Strong nitric acid hydrolyzes lactose and oxidizes both the primary alcohol and the hemi-acetal groups to carboxyl groups, with the formation of a very insoluble dicarboxylic acid called mucic acid. This is derived from galactose and is sometimes used for identification purposes.

Biochemically, lactose is not fermentable by ordinary bakers yeast, a fact sometimes used as a basis for determining milk and milk products in complex food products. Many bacteria, particularly the lactobacilli, ferment lactose into lactic acid.

The structural formulation postulates the existence of two forms of lactose—the alpha form, being more dextro-rotatory and with the –OH group in carbon-1 to the right or on the same side as the lactal ring, and the beta-form, being less dextro-rotatory and depicted as having the –OH group to the left. The existence of these two isomers is strong proof of the hemi-acetal structure. Actually, two forms are known and available. The alpha form is normally available as the monohydrate containing 5.0% water of crystallization, and is obtained from crystallization of lactose from solution at temperatures below 93.5° C. The alpha anhydrous lactose can be prepared by dehydrating alpha-lactose hydrate in vacuo at temperatures between 65 and 93.5° C., and is stable only in the absence of moisture. The beta-lactose is obtained from crystallization of lactose from solution at temperatures above 93.5° C.

The alpha and beta forms of lactose differ in their physical and in some of their chemical behaviors. For example, beta-lactose is sweeter,

more soluble and, according to Isbell *(6)*, oxidized at a faster rate by bromine to lactobionic acid, than is the alpha form. The specific rotation of alpha-lactose hydrate is + 89.4° and of beta-lactose, + 35°. The thermodynamic properties also are different.

In solution the two forms interconvert until an equilibrium is attained, irrespective of which form one starts out with. In the absence of catalysts and at ordinary temperatures, the rate of approach to equilibrium is fairly slow and can be followed by optical rotation, solubility, and rates of crystallization. When either form of lactose is added to water, the specific rotation changes gradually, due to the interconversion of the sugar from one form to the other, until at equilibrium the specific rotation becomes + 55.5°. This phenomenon is referred to as mutarotation and is common to many reducing sugars. Since specific rotation is an additive property, the composition of the mixture at equilibrium or at any time can be calculated. Sharp and Doob *(7)* have applied this principle in a quantitative method for determining the proportion of alpha- and beta-lactose in dry milk products. Further, by applying the mass action law to the reversible reaction between the alpha and beta forms of lactose in solution, Hudson *(4)* developed the equation

$$k_1 + k_2 = \frac{1}{t} \log \frac{r_o - r_\infty}{r - r_\infty}$$

which measures the rate of mutarotation. Here, the quantity $(k_1 + k_2)$ is referred to as the mutarotation coefficient, r_o is the optical rotation at zero time, r is the rotation at time t, and r_∞ is the final, or equilibrium, rotation. The equation expresses a first-order reaction. By plotting the difference between the rotation at time t and the equilibrium rotation against the time t, a straight line should result whose slope gives the mutarotation coefficient. Similarly, the equilibrium constants can be calculated from optical rotation by means of the equation

$$K = \frac{k_1}{k_2} = \frac{r_\alpha - r_\infty}{r_\infty - r_\beta}.$$

Another manifestation of the equilibrium of alpha- and beta-lactose in solution is in the solubility behavior of lactose. Hudson *(4)* found that when alpha-lactose hydrate was added in excess to water, with agitation, a definite amount would dissolve immediately and more would go into solution slowly, until a final solubility was attained. In the initial stage, the process is one of ordinary solution and is very rapid because of the large solid-liquid interface. In the second stage, the rate of solution is controlled entirely by the rate of conversion of the alpha to the beta form. Therefore, according to Hudson, the initial solubility is the equilibrium concentration, or true solubility, of the alpha form. The difference between the final and the initial solubility is the equilibrium concentration of the beta form. The mathematical expression for this relationship is given by the equation,

$$K = \frac{\beta}{\alpha} = \frac{S_\infty^{II} - S_o^{II}}{S_o^{II}}$$

where K is the equilibrium constant, S_∞^{II} is the final solubility, and S_o^{II} is the initial solubility. The final solubility can be determined without much difficulty. However, except at low temperatures, the measurement of initial solubility is complicated by the conversion of alpha-lactose to the beta form during the process of solution. The following equation

$$k_2 = \frac{1}{t} \log \frac{S_\infty^{II} - S_o^{II}}{S_\infty^{II} - S_t^{II}}$$

gives the rate of solution of alpha-lactose, referred to by Hudson as the maximum rate of solution, since the rate cannot be increased further by increasing the solid phase. This equation, likewise, expresses a first-order reaction, where k_2 is the rate constant and S_t^{II} is the amount of lactose dissolved in time t. Both k_2 and S_o^{II} can be evaluated by plotting log $\left(S_\infty^{II} - S_t^{II}\right)$ against t.

Several years ago, use was made of this equation in the development of a method *(1)* for the determination of alpha- and beta-lactose in dry milks. Briefly, the method involves determining the amount of lactose in solution at several time-intervals, when a mixture of dry milk and alpha lactose hydrate is continuously stirred in a known quantity of water at 25° C. Since the final solubility is known, by plotting the logarithm of the difference between the final solubility and the amount dissolved at time t against the time, a straight line is obtained whose intercept at zero time is log $\left(S_\infty^{II} - S_o^{II}\right)$, from which the term S_o^{II} can be calculated. The term S_o^{II} in this case includes not only the initial solubility of the alpha-lactose but also all of the beta-lactose in the dry milk sample. Since the initial solubility of alpha-lactose at 25° C. is known, the total beta-lactose in the dry milk can be calculated. The difference between the total lactose in the dry milk and the beta-lactose obtained gives the alpha modification.

The alpha-beta lactose equilibrium can be observed also in the crystallization of lactose from a supersaturated solution at ordinary temperatures. In a solution in which the concentrations of both alpha- and beta-lactose are in excess of their equilibrium concentrations, Hudson *(4)* found that the addition of

a large amount of fine crystals of alpha-lactose hydrate and application of vigorous agitation will cause the immediate crystallization of the excess alpha modification, followed by a slow conversion of the excess beta form to the alpha form, which then crystallizes as soon as it is formed. Since diffusion and nuclei formation are not important in this case, because of vigorous agitation and the excess of α-lactose hydrate crystals, the rate of crystallization is controlled entirely by the rate of transformation of the beta to the alpha form. This limiting rate, actually the rate of isomerization of beta to alpha, is described by the equation

$$k_2 = \frac{1}{t} \log \frac{Co - S^{II}_{\infty}}{C_t - S^{II}_{\infty}}$$

where k_2 is the rate constant and Co is the total initial supersaturated concentration of lactose after seeding with lactose crystals. Since the alpha lactose is already precipitated, Co is actually the excess beta plus the lactose in the saturated solution. In other words, the difference $\left(Co - S^{II}_{\infty}\right)$ is the excess beta-lactose in solution at zero time. Plotting of log $\left(C_t - S^{II}_{\infty}\right)$ which is the amount of beta-lactose at time t, against time t, gives a straight line, the slope of which gives a measure of the rate constant k_2, and the intercept at zero time gives the original excess beta-lactose in solution.

Advantage was also taken of this relationship in the development of an additional method *(2)* for the determination of alpha- and beta-lactose in dry-milk products. Briefly, the method consisted in adding a known quantity of dry milk mixed with an excess of alpha-lactose hydrate to a continuously agitated, saturated solution of lactose at 25° C. It was expected that the alpha-lactose from the dry milk, once dissolved, would immediately crystallize out and that the beta-lactose of the dry milk, once in solution, would undergo transformation to the alpha form, until the equilibrium condition was restored. Therefore, if the quantity of lactose in solution was determined at several time intervals and the log $\left(C_t - S^{II}_{\infty}\right)$ was plotted against the time t, a straight line would result. The intercept would give the beta-lactose. The difference between the total and the beta would give the alpha modification.

Beta-lactose also exhibits an initial and final solubility. Equations *(5)* similar to those presented above have been derived to describe the equilibrium and kinetics when beta-lactose is the solid phase.

The above discussion shows that the phenomenon of mutarotation, the slow changes in solubility, and rate of crystallization all are manifestations of the interconversion of alpha- and beta-lactose in solution. This is supported by the fact that both rate constants and equilibrium constants derived by the three methods are in agreement. Furthermore, all three equilibria are affected in the same manner by catalysts. Results obtained by Troy and Sharp *(8)*, showing the effect of pH on mutarotation, solubility, and crystallization, are given (Figure 2). The curves are of the same shape and indicate that the rate of interconversion of alpha- and beta-lactose is greatly accelerated by both acids and bases. In addition to acids and bases, anions such as phosphate and citrates also have great effects on the velocity of the reaction, which cannot be attributed to pH.

At the beginning of this discussion, it was brought out that the transition point for alpha-lactose hydrate and beta-lactose is 93.5° C. Below this temperature, alpha-lactose is the stable form crystallizing from solution; above this, the beta-lactose is the stable form. Figure 3 is a graph taken from the publication of Hudson *(5)*, showing the solubility relationship of the two forms of lactose. Starting with alpha-lactose hydrate as the solid phase, the final solubility at different temperatures is given by the curve labelled as S^{II}_{∞}. Similarly, with beta-lactose as the solid phase, the final solubility at different temperatures is expressed by the curve labeled as S^{A}_{∞}.

It will be noted that up to 93.5° C., the solubility of beta-lactose is much greater than that of the alpha-lactose hydrate; whereas, above that, the reverse is true. At the transition point, where the two curves intersect, the final solubilities of the two sugars are the same. As indicated before, the final solubility is the sum of the initial solubility of one form and the equilibrium concentration of the other form. Therefore, starting with alpha hydrate as the solid phase, the initial solubility, which is the true solubility of alpha-lactose, is represented by the curve S^{II}_{o}. The difference between the final solubility and the initial solubility, that is, curve S^{II}_{∞} minus curve S^{II}_{o}, gives the equilibrium concentration of the beta sugar, which is represented by the curve C_A. Similarly, starting with beta-lactose as the solid phase, the initial solubility, which is the solubility of the beta form, is represented by the curve S^{A}_{o}, and the equilibrium concentration of the alpha form is represented by the curve C_H.

This diagram gives a simple explanation of the fact that below the transition point the alpha hydrate is the stable form, but above it, the beta anhydrous lactose is the stable form. Consider, for example, a mixture of beta-lactose and water at 60° C., the beta dissolving to form a staturated solution whose concentration is indicated on the curve S^{A}_{∞}, which has a value of about 55% at 60° C. Now, the concentration of the alpha form in this equilibrium mixture is represented by the curve C_H, and it can be seen that this curve at 60° C. is above the

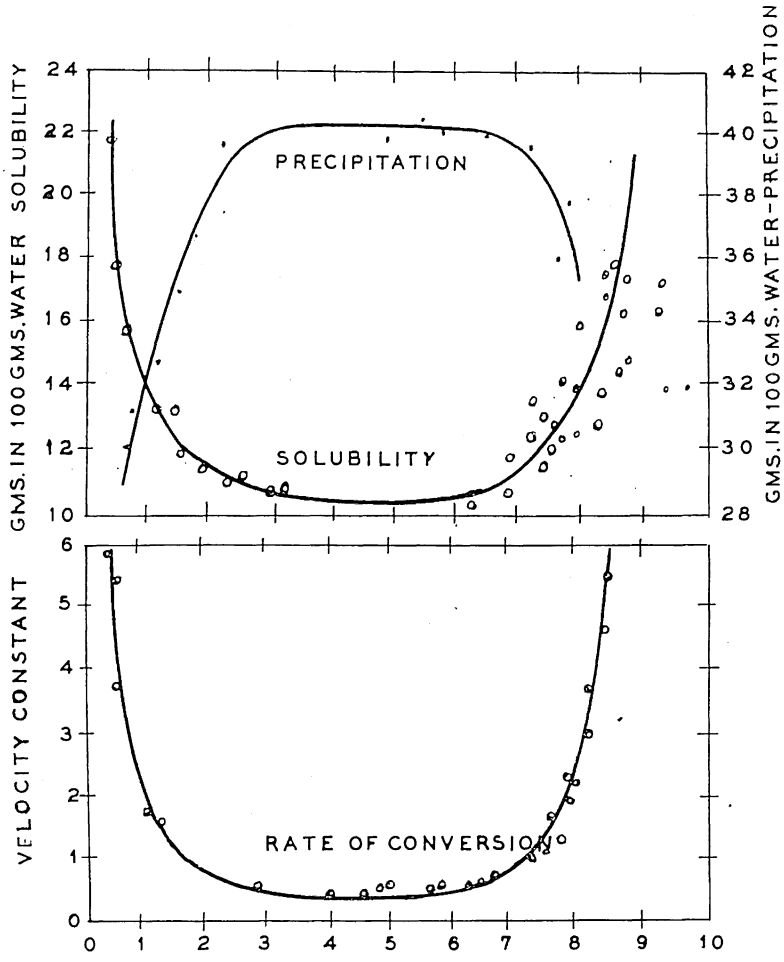


FIG. 2. The effect of pH on the rate of change of the forms of lactose into each other as influencing the rate of solution and precipitation of lactose. (Data of Troy and Sharp.)

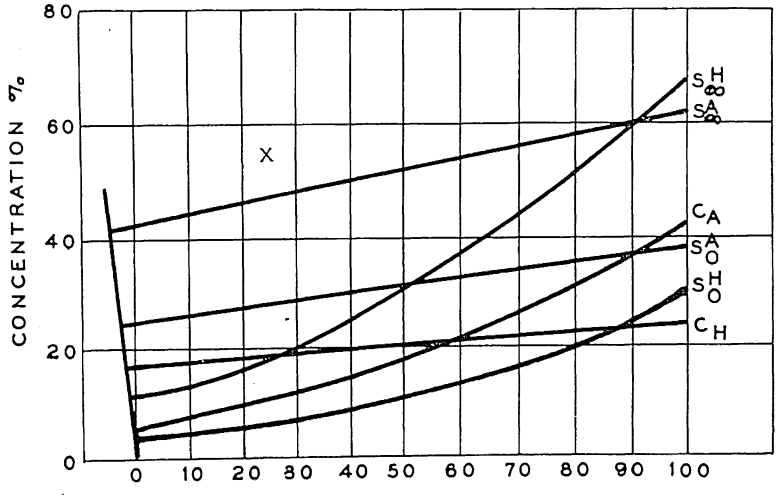


FIG. 3. The solubility relations of lactose. (Data of C. S. Hudson.)

initial solubility of alpha-lactose represented by S_o^{II}. Therefore, in this solution, the alpha hydrate is in excess and will crystallize out. Above the transition point, the alpha hydrate is more soluble than the beta form. At 100° C., for example, a saturated solution of alpha-lactose hydrate will give an equilibrium concentration of beta-lactose, represented by the curve C_A, which is above the initial solubility of beta-lactose. Therefore, beta-lactose will crystallize out. At the transition point, the final solubilities of both alpha- and beta-lactose are equal. Both alpha- and beta-lactose are saturated and either form can crystallize from solution at this temperature.

Sometimes it has been observed that beta-lactose can crystallize out at room temperature, which may sound somewhat surprising, since the transition point is 93.5° C. That this is possible can be seen easily from Fig. 3. Take, for example, the point X, which is supersaturated with respect to both alpha- and beta-lactose. If such a solution is seeded with beta-lactose, this form can crystallize out from solution until it reaches the final solubility of beta-lactose, represented by the line S_∞^A. However, at this concentration, the solution is still highly supersaturated with respect to the alpha form. Therefore, if conditions are favorable, the alpha-lactose will finally crystallize out as the hydrate, until the solution reaches a composition represented by the line S_∞^H.

In general, the rate of crystallization of lactose, or of any material, depends on, *1)* the degree of supersaturation, *2)* the surface area of the crystal or the number of nuclei, and *3)* the viscosity of the solution which, in turn, governs the diffusion of the solute molecule to the crystal surface. When crystallization of lactose takes place in the presence of numerous crystals and with agitation, nuclei formation and diffusion are probably not important. The conversion of beta- to alpha-lactose is usually the limiting rate. In the absence of nuclei and agitation, lactose solution can be highly supersaturated without crystallization. The so-called supersolubility value appears to be fairly definite. When the amount of lactose in solution exceeds the supersolubility value, spontaneous crystal formation will, of course, take place. At ordinary temperatures, the alpha-lactose hydrate is the form which usually crystallizes from supersaturation. Since there is a large amount of alpha-lactose in solution to start with, the rate of crystallization in the initial stage may be entirely dependent upon the number of nuclei or the viscosity of the solution. For example, Herrington *(3)* has found that the tendency to form crystals passes through a maximum as the supersaturated solution is cooled. Whittier and Gould *(10)* have found that the best temperature to crystallize lactose is 30° C., where the velocity is the highest. However, in the later stage of crystallization, it is reasonable to assume that the rate of interconversion of alpha- and beta-lactose is the limiting rate.

Because of the relative stability of supersaturated lactose solution, it is possible to prepare a solid supersaturated solution of lactose, generally referred to as lactose glass. This is done by quickly removing most of the water from a highly concentrated solution so that neither the alpha nor the beta form has a chance to crystallize. Therefore, in lactose glass the proportion of alpha- and beta-lactose is close to the equilibrium proportion. The subject of lactose glass undoubtedly will be discussed further, in the papers to follow.

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PROBLEMS OF LACTOSE CRYSTALLIZATION IN CONCENTRATED MILK PRODUCTS[1]

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Lactose crystallization always occurs during the processing of sweetened condensed milk products; usually occurs in the manufacture of condensed and dried wheys, and may take place in such products as ice cream, plain condensed, dry, and frozen milks. The crystallization more often happens, or becomes sensible, during the holding periods following manufacture.

The fundamental reason for lactose crystallization in all cases is either an insufficiency of water to hold it in solution under the prevailing conditions or sufficient water to furnish a labile concentration when lactose is in the amorphous or glass state.

The presence of lactose crystals in dairy products constitutes a defect when their size is such as to create a mealy, sandy, or gritty texture; when they tend to settle and form a deposit; when their presence changes the physical character of the product or any of its ingredients, or when they interfere with use of the product.

In some dairy products, lactose crystallization can be prevented. In others, this is impossible or inadvisable, so that attention is necessarily focused on the limitation of crystal size to insensible magnitudes.

Sweetened condensed milk. The necessity for control of lactose crystallization in sweetened condensed milk has been recognized from the beginning, and this product served to focus the attention of scientists on the idiosyncrasies of milk sugar in the processing and storage of dairy products.

As sweetened condensed milk comes from the evaporators, the lactose is at, or near, the saturation point. Eventually, when cooled to 60 or 65° F., between two-fifths and two-thirds of the lactose present will emerge as crystalline α-lactose hydrate. This happens because milk sugar is soluble to the extent of only about 15 parts to 100 parts of the water as found in this product *(11)*. Actually, there are 40 to 47 parts of lactose per 100 of water in sweetened condensed milk of average composition, made up of an equilibrium mixture of about 40% α form and 60 per cent β form. Crystallization of lactose, therefore, can not be prevented, short of previous hydrolysis, and desirable texture must be achieved by limiting the size of the crystals. To obtain smooth texture, it has been shown *(23)* that a narrow range of crystal-size is necessary, averaging from about 10 to 11 μ along the longest edge. At this size, there are between 200,000 and 300,000 crystals per ml. *(23)*.

Theoretically, small crystals and a multitude of them result when a rather highly supersaturated solution is seeded under conditions of vigorous agitation. Lactose readily forms supersaturated solutions. In common with other sugars, it manifests a reluctance to crystallize, especially in the absence of agitation, until the excess sugar in solution is at a very high level *(7, 30)*. It has been noted, however, that too great supersaturation actually reduces the crystallization pressure and slows the mass formation of crystal nuclei *(31)*. Moreover, in sweetened condensed milk the viscosity increases rapidly with reduction of temperature, and interferes with diffusion to the crystal-forming centers. Experiment has shown that the greatest number of smallest crystals is obtained when crystallization is forced by seeding at temperatures between 80 and 95° F., depending on the composition of the sweetened condensed milk and its viscosity *(10, 31)*. Several texts contain data showing the final solubility and the spontaneous crystallization curves for lactose *(1, 3, 10, 32)*. The area between these curves defines the metastable zone, where forced crystallization should be practiced for most desirable results. Forced crystallization consists of adding 4 to 8 oz. of impact-pulverized α-lactose hydrate dust, per 1,000 lb. of vigorously agitating product, in such a way that maximum dispersion results, and of continuing agitation for from 30 to 60 min. without reduction of temperature.

Since the final cooling temperature of sweetened condensed milk is 60 to 65° F., it should be appreciated that more lactose will crystallize on the nuclei already formed as the temperature is reduced following forced crystallization. This occurs not only because of the decreasing solubility of the α-lactose still in solution, but because of the equilibrium relationship between the α and β forms, which results in mutation of the β form to α, as the α in solution is decreased by crystallization. This mutation or reversion is a relatively slow process. At 60° F., it is only about 90% complete after 8 hr. *(8, 9)*.

[1] Authorized as Paper No. 2189 in the Journal series of the Pennsylvania Agricultural Experiment Station.

and for this reason a period of agitation is desirable between final cooling and packaging of the finished product. This agitation assists in accelerating the β to α reversion, and in making uniform the crystal growth in a product as viscous as sweetened condensed milk.

While many commercially used types of cooling and crystallization equipment for sweetened condensed milk make use of the forced crystallization concept at the optimum temperature, a notable exception is the long and successfully used submerged coil, or internal-tube, method, where milk from the evaporators is pumped continuously through the cooling tubes to bring it to its final temperature, with or without previous seeding. It appears that in such equipment seed crystals are encountered in the cooler when the temperature drops far enough to insure a satisfactory degree of lactose supersaturation. The crystallizing impulse is disseminated through the mass of milk as it is forced in a rolling motion through the tubes. Inasmuch as there is no lengthy forced crystallization period in this type of cooling, the desirability of a lengthy period of agitation before filling would seem to be emphasized.

Some manufacturers, who practice standardization of overconcentrated milk by adding sterile water before filling, feel that a secondary advantage of this practice is the reduction in size of the lactose crystals by partial solution in this water. No studies substantiating this have been reported.

The sedimentation of lactose crystals, in a given batch of sweetened condensed milk during holding, is a function of crystal size, viscosity, and time. If crystallization techniques are satisfactory, sedimentation can best be avoided by adjusting the viscosity, by means of preheating conditions and milk-solids-not-fat level, keeping in mind the thickening tendency on aging and the probable time and temperature of storage. Variations in ambient temperature during storage (heat-shock) are known to cause growth in crystal size by solution of small crystals, and subsequent increase in size of the larger ones, as the temperature changes vary the solubility of lactose.

Other highly concentrated products in which lactose will crystallize during or following manufacture, are generally treated like sweetened condensed milk, utilizing a seeding procedure and forced crystallization to assure small crystals which will not grow to objectionable size.

Some concentrated whey products are processed in this fashion, and sweetened condensed whey and preserved concentrated ice cream mix always are so treated. The latter product, however, may include some sodium caseinate as part of the serum solids, to reduce the level of lactose present *(32)*.

Plain condensed milk. Crystallization and sedimentation of lactose in plain condensed milks are not common, but occasionally do occur with high-solids products. Some crude observations have indicated that 4-to-1 whole milk will usually crystallize under unagitated, 40° F. holding in 72 hr.; similar skimmilk, not until after 240 hr. The former product contains about 40 parts of lactose to 100 water, and the latter about 31 parts. At 3-to-1 concentration, where the highest lactose : water ratio is about 24 to 100, usually neither whole nor skimmilk will show evidence of crystallization up to 480 hr. These results agree in general with those which have been made on supersaturated water solutions of lactose *(7)*. It probably can be said that lactose will not crystallize in plain condensed milk during customary, unagitated holding periods in refrigerated storage, so long as the lactose : water ratio is not over 30 parts per 100—unless inadvertent seeding or some other crystallizing impulse is furnished.

Some milk-drying plants, using continuous methods, have experienced trouble with lactose crystallization in the accumulation tanks feeding the driers. These tanks are often uninsulated and unagitated, and considerable cooling may occur in quiescent areas, increasing the degree of supersaturation of the lactose to a point where spontaneous crystallization may take place. Once this has happened, crystallization builds up rapidly, because of the seed material, and a sludge may develop. It has been necessary in some cases to suspend operations for cleaning, or to divert the milk to an auxiliary tank until remedial action can be taken. Knowledge of this problem is meager, but some available information indicates that when the temperature of the milk is uniformly maintained by agitation at 120° F. or higher, no crystallization occurs. This appears logical, inasmuch as little or no supersaturation would exist at this temperature. It also would seem possible to avoid the problem by limiting the solids content of the skimmilk from the evaporators to 45%, for at this level the lactose : water ratio would be 45 to 100, corresponding to a spontaneous crystallization temperature of 70° F. or less.

Ice cream. The crystallization of lactose in ice cream, causing sandy texture, is a recurring problem to manufacturers. Evidence indicates that in freshly hardened ice cream the equilibrium mixture of the α and β forms of lactose is in the glass state, and is stable as long as the temperature remains at the hardening-room level *(7, 22, 27)*. In the freezing of ice cream, the lactose solution apparently passes through the labile zone so rapidly and at so low a temperature that no opportunity exists for the molecules to diffuse and orient into crystal structures. Undoubtedly, the high viscosity of the unfrozen liquid is a crucial factor in this connection *(22)*.

When ice cream is warmed to, and held at, dipping temperatures or dispensing-cabinet temperatures, some ice melts, and there must be produced an infinite variety of lactose concen-

trations, over a period of time, as molecules diffuse slowly into these water droplets from the amorphous glass. Some of the concentrations doubtless will be in the labile zone for the temperature, and permit spontaneous crystallization; others will be in the metastable zone, where crystallization can occur if a stimulus in the form of lactose crystal nuclei, or fine particles of extraneous matter, exists. Because of the low temperature, crystallization pressure will be low and no mass crystallization would be expected. Nevertheless, the nuclei formed will act as a seed impulse for further crystallization, as opportunity offers, and will themselves tend to grow slowly with time. Eventually, the ice cream will become sandy.

Since all ice cream, as conventionally made, contains more lactose than is soluble in the available water at the temperature, the wonder is that all ice cream does not become sandy after a few days in the dispensing cabinets. The fact that limitation of the serum solids content of the mix prevents, or at least greatly delays, lactose crystallization can not be entirely explained on the basis of our present knowledge. However, it is true that with low lactose mixes the sucrose : lactose ratio is generally greater, and at comparable temperatures the lactose-water concentration would be lower in such mixes.

An equation has been proposed *(22)* for calculating a safe maximum serum solids content for ice cream mixes. This establishes an average limit of 14.5% for the serum solids content of the serum, but may be varied about 1%, down or up, depending on whether conditions will be conducive or nonconducive toward the development of sandiness in the ice cream. Experience has shown that this method of controlling the defect is generally, but not absolutely, satisfactory.

Fruit and nut ice creams are more prone to become sandy than most plain flavors. The usual explanation is that the added flavor substances either furnish fine particles capable of acting as crystallization nuclei, or that they absorb water from the serum.

Some manufacturers have used high levels of serum solids and avoided the problem of sandiness by employing skimmilk products from which substantial proportions of the lactose have been removed. Others have utilized, for this purpose, proteins (usually sodium caseinate) separated from milk products.

A method of obtaining high serum solids ice cream without texture problems was patented in 1932, but has not been used much commercially *(6)*. In this procedure, a substantial portion of the total serum solids is added in the form of dry skimmilk to the partially frozen ice cream. It was claimed that inasmuch as the lactose of the dry product can not go into solution, because of the already supersaturated condition of the ice cream, it therefore can not crystallize later. This reasoning seems fallacious, in view of the fact that lactose in the added dry milk is in the same glass state as that in the hardened ice cream. If the latter is capable of crystallizing with a rise in temperature, there seems no good reason why the former should not do so under dispensing-cabinet conditions. Nevertheless, this method does seem to accomplish the purpose in view.

More recently, enzymatic hydrolysis of the lactose of skimmilk has attracted some attention as a means of lowering the lactose level of ice cream mixes *(19)*, but the time element and inconvenience would seem to militate against its large-scale adoption.

A different approach to the problem of sandiness which is of interest was patented in 1953 *(4)*. This method is reminiscent of the psychology: "If you can't beat 'em—join 'em." It takes the viewpoint that since lactose crystallization is virtually impossible to prevent absolutely, it is better to force crystallization to occur and to control the size of crystals, as is done with sweetened condensed milk. The method has been studied in considerable detail at California *(13, 14, 15)*. When ice cream mix, immediately before freezing or in the partially frozen state, is properly seeded with fine lactose dust (3 to 7 μ in particle size), mass formation of crystal nuclei occurs. The crystals are so small and so numerous that they never reach sensible size, even though considerable quantities of β-lactose slowly revert to the α-form and build up on them during the holding period. Ice cream containing 18% serum solids has been produced by this procedure and held for 10 mo. at 12° F. without evidence of sandy texture. It has also been demonstrated that dried products, such as dry skimmilk and dry whey, added at the freezer, induce mass crystallization in a manner similar to that of the seed lactose *(15)*. It appears, therefore, that the earlier patent covering the addition of dry skimmilk at the freezer actually avoids sandiness, not because the lactose of the dried product fails to go into solution, but because it induces mass crystallization in the form of impalpable crystals. This method of overcoming the problem of sandy ice cream deserves more study, and its possible effect on body, texture, and melt-down characteristics should be investigated.

Frozen milk. In frozen milk, it is the loss of stability on the part of the caseinate that determines the length of the period of satisfactory holding *(5)*. It is of interest, and possibly highly significant, that a relationship between casein flocculation and lactose crystallization has been demonstrated in frozen concentrated milk at the National Dairy Research Laboratories *(29)*. This has been nicely confirmed and extended by studies at the National Research laboratories, Ottawa, Canada *(17, 18, 25, 26)*.

In 3 : 1 concentrated skimmilk, preheated at temperatures below 170° F. and held in frozen storage, the casein maintains its stability

as long as the lactose remains as a supersaturated solution, or glass. At temperatures of — 20° F. and below, this condition seems more or less permanent, and milk can be held for very long periods. Between — 10° and 0° F., lactose crystallizes rather reluctantly and only after a considerable induction period. Above 0° F., however, it is only a matter of a few days before lactose appears in crystal form, and soon thereafter casein is found in the flocculated condition on defrosting.

Incidentally, the similarity between lactose crystallization in frozen milk and in ice cream is close, as might be expected. First, the temperatures at which crystallization occurs, or does not occur, are very much alike in the two products. Secondly, just as a reduction in the serum solids content of ice cream mix acts to postpone the development of sandiness, so does the lowering of the concentration ratio of the concentrated milk, prior to freezing, delay crystallization in the frozen product. This is in spite of the fact that lactose concentration in both frozen products is dependent not on composition—but on temperature. For instance, it has been shown that when fluid milk, and the same milk concentrated to 30% solids, were each frozen and held at 15° F. for 23 days, the former exhibited no lactose crystallization and no destabilization of the casein; whereas, in the latter, 75% of the lactose had crystallized and the casein was in an advanced stage of flocculation *(28)*. Thirdly, the melt-down of sandy ice cream almost always exhibits a curdy character, which is doubtless a result of casein flocculation similar to that found in defrosted, crystallized frozen milk.

There is also a kind of analogy between the destabilization of casein in frozen concentrated milk when lactose crystallizes, and the loss of solubility and dispersibility of casein in dry milk when lactose crystallization and lumpiness occur, following absorption of moisture. Both seem to become manifest as a result of, or at least subsequent to, lactose crystallization. This analogy, however, seems not to hold in the instantizing treatment of dried milk.

Why, or by what means, the change of lactose from a molecularly dispersed state to a crystal form influences the physical or physico-chemical properties of calcium caseinate, is not known, but it may be pertinent to mention what is known relative to the nature of the casein floc as it appears in frozen milk.

The loss of dispersibility is noted on defrosting, especially on reconstitution. In the early stages, it is reversible with agitation and/or heat, but with continued storage it becomes irreversible. It is thought to result from a salting-out effect caused by the high concentration of solubles in the unfrozen water of the frozen product. Casein of similar characteristics can be obtained by aging skimmilk saturated with sodium chloride at room temperature *(5)*. Instability of the casein can be aggravated by heat denaturation of the whey proteins and by anything which stimulates lactose crystal nuclei formation prior to freezing *(18)*. Instability can be lessened by the presence of other sugars *(17, 33)*, by sequestration or removal of calcium ions *(33)*, and by enzymatic hydrolysis of a portion of the lactose prior to freezing *(28, 29)*.

It has been postulated that inasmuch as other sugars in solution manifest a stabilizing influence on the caseinate *(26, 33)*, lactose may act similarly *(29)*. When it crystallizes from solutioin, it leaves the unfrozen water with lower osmotic pressure, so that more water freezes and intensifies the salt concentration which in turn, operates to floculate the casein *(17)*. Sugars, furthermore, contribute greatly to the viscosity of the unfrozen liquid, tending to block diffusion and slow any of the changes in the system which affect casein stability. There is also a possibility that sugars form compounds with calcium *(7)*, an ion to which casein is particularly sensitive.

This newly discovered relationship between lactose nucleation and casein instability in concentrated milks deserves further study.

Dried milks. Lactose does not normally crystallize in the drying of milk by the usual spray and drum methods. It is found as an equilibrium mixture of α and β forms in the amorphous glass state *(27, 32)*, in fresh powder, and will so remain if the product, which is very hygroscopic, is protected from moisture.

Lumping and caking of dried milk after manufacture is generally conceded to be caused by the gradual absorption of moisture, which dilutes the lactose glass to the point where a supersaturated solution in the labile zone is obtained. Crystallization of α-lactose hydrate usually results, and the masses of growing crystals form a structure causing lumpiness and, eventually, solidification *(27)*. β-lactose also may take part in this crystallization, when conditions are favorable *(20)*. The decrease in solubility which usually accompanies lumping may be due to the loss of casein stability, as noted in frozen concentrated milk, when lactose crystallizes *(29)*. Air-tight packages are essential in preventing crystallization and in maintaining the quality of dry milk.

The process of producing instantly soluble dry milk by humidifying the freshly dried product, clustering the particles and redrying, permits partial crystallization of lactose before the moisture level is again reduced below the point where crystals can form. Such instant milk contains about three parts α-lactose to two parts β-, instead of the usual two α- and three β- *(2)*. Lactose crystallization is apparently not necessary for the improved dispersibility, inasmuch as instant milk made by other techniques, where the equilibrium ratio of α- and β-lactose is not disturbed, shows good dispersion characteristics, at least when freshly made *(2)*.

Dried whey. The drying of whey is complicated by the fact that lactose forms such a large fraction of the solids. When dehydrated, whey is much more hygroscopic than skimmilk, easily becomes sticky, and is difficult to handle.

It is possible to reduce the difficulties by mixing the whey with fillers, such as various flours, or even skimmilk *(12, 24)*, before drying, but this changes the composition and adds to the bulk of the finished product.

A number of special processes *(10, 32)* have been patented for manufacturing stabilized dried whey. A stabilized product is one which will not absorb objectionable quantities of moisture from the air and become sticky, lumpy, or solid. Usually, stabilization is accomplished by causing a considerable proportion of the lactose to crystallize from the concentrated product before the final moisture is removed; or by rehumidifying the dried product to cause crystallization, with or without secondary drying. Usually, the crystallized lactose is the α-hydrate, but if the crystallization is forced at temperatures above 200° F., β-anhydride crystals result *(21)*. In either case, if sufficient lactose is converted from the glass state to the crystal state, the dried whey will be relatively nonhygroscopic and easily handled and used. These methods require special equipment and special procedures which are foreign to the conventional methods of drying fluid whole and skimmed milk.

Conclusion

In conclusion, it can be said that lactose is troublesome in processing, packaging, and storage of many concentrated milk products, because it tends to crystallize. Although many techniques for controlling and minimizing the difficulties of crystallization, and threatened crystallization, have been developed, the problem is not completely understood and some of the corrective measures now employed are only partially effective. Research in this area should be fruitful, and the results useful in improving product-quality and in prolonging keeping periods.

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COMMERCIAL PRODUCTION OF LACTOSE

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There have been so many excellent papers published within the last 15 yr. on the methods of manufacture of lactose that a review of this literature would be repetitious. However, it might be desirable to refer to some of the more important reviews. Webb and Ramsdell *(7)*, Webb and Whittier *(8)*, Whittier *(10)*, and Whittier and Webb *(11)*, have published excellent reviews of the subject. Those by Hunziker *(5)*, Kastens and Baldauski *(6)*, and Weisberg *(9)* should also be included.

These papers have been very helpful to the dairy industry. Those from the Bureau of Dairy Industry *(7, 10)*, published during World War II, undoubtedly were responsible for eliminating a considerable amount of confusion, as well as saving much time and product, when the production of penicillin caused an increase of more than 200% in the production of lactose within a 3-yr. period.

Figure 1 gives the annual production of crude lactose in the United States from 1943 to 1955. This figure shows the effect of penicillin upon lactose production. Prior to 1944, lactose had been produced at a level similar to that of 1943, at approximately 7.3 million lb. When it was found that lactose increased the growth rate of *Penicillium notatum*, resulting in large increases in the yield of penicillin, then the lactose production curve followed that of penicillin. The peak production figure of 50,156,000 lb. in 1951 has been reported to be due to the combined effect of the Korean war and the use of penicillin as a growth stimulant in animal feeds. The curve for the last 3 yr. is still ascending at a rate comparable to that of the 1943-1948 period. It is also evident that the production of 35,356,000 lb. for 1955 is about a fivefold increase during a 12-yr. period.

It is estimated that about 10 billion lb. of whey are obtained yearly in the United States from cheese and casein manufacture. Assuming that there is 5% lactose in whey and that 60% of it is recovered, then less than 7% of the available supply is used for this purpose. Since it is desirable to have many outlets for products which have not been fully utilized, it is of interest to explore some of the reasons why so little whey is used for lactose production. The probable reasons are:

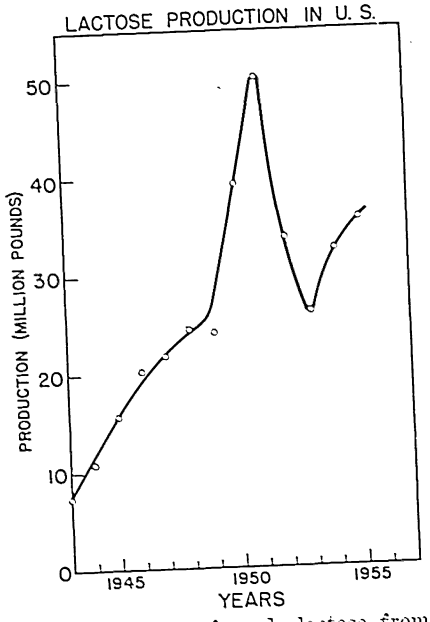


FIG. 1. Production of crude lactose from 1943 to 1955.

1. Need for more uses for lactose.
2. Need for better methods of obtaining lactose.
3. Need for more uses for the lactose mother liquor in the making of edible or pharmaceutical products.

The last two needs will be discussed in the

remaining part of this paper. The first point, relative to the need for more uses for lactose, will be discussed in the next paper in this symposium, The Commercial Uses for Lactose.

We feel that better methods of producing lactose will be found, in which a high yield of edible lactose will be obtained by one crystallization and leaving as a by-product the lactose mother liquor, which will be used as a highly desirable source of edible proteins and salts. The advantages of such a method may be seen after describing some of the procedures relied upon by many methods of obtaining lactose.

These methods, in the main, rely upon the removal of the proteins, before concentrating the whey. This is carried out as follows:

1. Lime or calcium salts added to whey.
2. Whey heated to near boiling to coagulate whey proteins, followed by decanting and filtering prior to concentrating to about 40% total solids.
3. Partially concentrated whey removed from vacuum pan, boiled with decolorizing carbon, filtered and then concentrated to about 70% total solids.
4. Lactose crystallized, followed by removal from mother liquor by centrifuging.
5. Lactose yield between 60 and 70%.
6. Lactose mother liquor and the two coagulated whey protein fractions used for animal feed.

Bell, Peter, and Johnson *(2),* of the Bureau of Dairy Industry, developed a method in 1928 in which the whey proteins were left in the whey during the lactose removal. This protein was found to be in a more or less soluble state in the mother liquor. Almy and Hull *(1)* found that by adding the calcium-sequestering agent, sodium tetraphosphate, they could improve the method of Bell, Peter, and Johnson. It appeared that the sodium tetraphosphate prevented the high concentration of calcium salfts from coagulating the whey proteins that interfered with good lactose crystallization and centrifuging. This method has been found to be very practical for obtaining a high grade of edible lactose. Yields approaching the theoretical have been obtained, and the resulting mother liquor has been found satisfactory for making a sweetened condensed product that has been used in confectionery products *(3).*

A further advance in methods for making lactose has been found in the use of the exchange resins. Hull *(4)* was able to exchange the calcium in whey for sodium by a base exchange resin and to increase the yield of lactose. After the calcium was decreased, it was found that the whey proteins were quite resistant to heat denaturation, so that they did not interfere wtih lactose crystallization or centrifuging. Consequently, the lactose yields were high and the ash and protein contents of the edible lactose were very low. The mother liquor is a very desirable product for edible uses, because the whey proteins are in a soluble, undenatured state.

Summarizing, it should be evident that every effort should be made to adopt new methods and techniques that will enhance the value of all the dairy products and by-products. If the by-products that have been considered either as animal feeds or as waste products can be re-evaluated and found to have a place as food for humans, the whole dairy industry will be benefited.

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UTILIZATION OF LACTOSE

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In cows' milk, lactose is the largest single solids component, with the possible exception of fat in certain breeds. In some cases, dairy products may be used largely because of their lactose content, and at other times the desired effect comes about from a combination of the lactose and other constituents of milk. However, in discussing the uses of lactose, only lactose which has been separated from the other constituents will be considered. In commerce today there are four main grades of lactose available: Lactose Fermentation Grade, Crude Milk Sugar, Lactose Edible, and Lactose U.S.P. The term Technical Grade has been used in the past, but this designation now has largely given way to Lactose Edible. Recently, a Lactose U.S.P Spray Process has come into use. In degree of refinement, it is intermediate between Lactose Edible and Lactose U.S.P. made by the conventional process. For comparative purposes, a typical analysis of each grade is listed (Table 1).

Each grade shown represents a different degree of refining or purification. As the impurities are removed, there is usually a corresponding increase in cost. In commercial practice the grade used for any particular application is usually the lowest-cost product which will still satisfy the requirements in question.

Not only is there competition of one grade of lactose with another, but to a large degree lactose competes with other sugars for specific usages. The unique physical and chemical properties of lactose make it superior to other sugars for certain applications, but in other respects it cannot compete. Obviously, when one is looking for new uses for lactose, the most fruitful areas are those applications which take advantage of the unique properties of it, or at least those applications where a premium may be paid for certain characteristics in which lactose may excel. Research into new uses or applications is often rewarding but may be disappointing. At times, the disappointments can be turned into profitable alternate uses.

A few of the more obvious differences between lactose and common sugar, or sucrose, are: *(1)* Sweetness. Using a scale of 100 for sucrose, lactose has a sweetness of only 16 or, in other words, it is only about one-sixth as sweet. *(2)* Lactose is much less soluble in water. At room temperature a saturated solution contains only about 20%. *(3)* Lactose is not fermented by bakers' yeast. *(4)* Lactose excels in the retention of flavors and aromas. Most dairymen are well aware how fluid milk will take on the flavors of the surroundings if it is not protected. This characteristic comes largely from the lactose. *(5)* Lactose is also superior in absorbing color and pigments. *(6)* When heated in the presence of proteins, lactose will turn brown, or caramelize. This is often a vexing problem with certain milk products, such as evaporated milk, but this characteristic is taken advantage of in many applications or uses of lactose. *(7)* Lactose is believed to have special nutritional advantages, compared with other sugars.

TABLE 1

Typical analyses of various grades of lactose[a]

	Lactose Fermentation Grade	Crude Milk Sugar	Lactose Edible	Lactose U.S.P., Spray Process	Lactose U.S.P.
Lactose, by difference *(%)*	97.00	97.75	98.75	99.25	99.60
Free moisture *(%)*	0.50	0.40	0.60	0.50	0.20
Acidity, as lactic *(%)*	0.40	0.30	0.04	0.03	0.03
Protein (N×6.25) *(%)*	1.00	0.75	0.20	0.10	0.05
Ash *(%)*	0.80	0.65	0.15	0.05	0.03
Fat (ether extractable) *(%)*	0.30	0.20	0.15	0.10	0.05
Heavy metals, as Pb *(p.p.m.)*	5	3	2	1	1
Turbidity (3 g.+10 ml.) *(p.p.m.)*	.....	.....	30	10	3
Color (3 g.+10 ml.) *(p.p.m.)*	.....	.....	30	25	15
Scorched particles *(mg. per 50 g.)*	22.5	15.0	7.5	7.5	7.5
Bacterial estimate *(per gram)*					
Standard plate count	.....	.....	1,000	1,000	1,000
Escherichia coli (in 10 mg.)	.....	.....	Negative	Negative	Negative
Sporeformers *(in 100 mg.)*	.....	.....	Negative	Negative	Negative

[a] The magnitude of the values given depends somewhat upon the analytical methods used. This is especially true of the free moisture values. Some moisture methods do not differentiate between free moisture and water of crystallization.

These and other characteristics of lactose will be discussed in more detail as specific uses are mentioned.

Fermentation Media. Lactose is the preferred carbohydrate in many fermentation media. This was especially true in the early production of penicillin by mold fermentation. It is fermented more slowly than many other sugars. Someone compared its utilization in a fermentation substrate to a slow-burning oak stump, as contrasted with pine kindling. With penicillin urgently needed for defense purposes in the early days of its production, cost became an item of secondary consideration, especially since many producers worked on a cost-plus basis. After the war, research workers found that fast-fermenting sugars such as glucose could be fed continuously into fermentations as the sugars were depleted and, in so doing, replace slow-fermenting lactose in the medium. This practice resulted in a reduction in the tonnage of lactose used for certain fermentations, but many companies still prefer to use lactose despite this innovation. Improvements in the efficiency of the manufacture of lactose in recent years have resulted in lowering costs, especially of the unrefined fermentation grade. This has encouraged the continued use of lactose as the carbohydrate of choice in many commercial fermentation media. The fermentation usage probably accounts for the largest single outlet for lactose in the industry today.

Pharmaceutical. The pharmaceutical and drug trades have used refined lactose for many years. The principal use is as a diluent or extender for many drugs. A common practice has been to mix or grind powdered medicinals or drugs with lactose to produce what is known as triturates. Lactose has peculiar characteristics which favor tablet- or pill-forming, both by compression and by molding. In making molded tablets a liquid, usually a volatile one like alcohol, is mixed with the lactose carrier and active ingredient or ingredients to make a paste which can be molded. After drying, the tablets are firm. Lactose is also used as a coating for some pills. For all of these applications a relatively high purity lactose is used. It must meet the requirements or specifications set forth by the United States Pharmacopeia; hence, is known as Lactose U.S.P.

Lactose is a common ingredient in many bacteriological media. It is used especially in the detection of coliform organisms for, unlike most other organisms, they will ferment lactose with the production of gas.

Infant foods and special diets. Bottle-feeding of babies has become very popular and many milk-based preparations are sold for this purpose. It is lamentable that some early specialists in pediatrics did not recommend lactose as an ingredient for infant feeding and actually campaigned against it. They maintained that lactose, or its break-down products, in the intestinal tract caused gastric inflammation and injury to the baby. There is still room for research into the role of lactose in nutrition, especially for infants, but at least many of the prejudices of a few decades ago seem to have disappeared. The attitude seems to be that Providence must have had good reasons for putting lactose in the milk of all mammals and, further, that it might not be just an accident that mothers' milk is much higher in lactose than cows' milk. Today most of the proprietary formulas for bottle-feeding babies are fortified with lactose and this market has become an important one.

When lactose was first used in infant foods, the grade used was largely Lactose U.S.P. Basically, it is uneconomical to refine lactose to contain less than 0.1% minerals and then add it to dry milks which contain about 8.0% minerals. The practice seems even less justified because the minerals removed from lactose originate in milk. Lactose Edible was developed largely for use in infant foods. This grade could be produced more economically than the U.S.P. grade; in addition, requirements such as bacterial content, etc. were beyond those called for by the Pharmacopeia. In this connection, it is of interest to note that the Lactose U.S.P. monograph makes no mention of bacterial count, coliforms, or spore-formers, which are considered so important in most food products. The specifications for Lactose Edible set up by various manufacturers in the industry recognize the importance of these items.

Beta-lactose has been popular for use in infant feeding. It is sweeter and more soluble than the alpha-monohydrate form of commerce, which is also the U.S.P. grade, and usually commands a premium in price. From the standpoint of nutrition there is little if any advantage to the beta form, for when either the alpha or the beta form goes into solution it forms the same equilibrium ratio of alpha to beta. Both dissolve before digestion—even if eaten dry. Dry beta-lactose is anhydrous, whereas the alpha-monohydrate contains about 5% of water of crystallization.

The galactose half of the lactose molecule seems to be responsible for many of the nutritional advantages claimed for lactose. It is reported to aid in the development of cerebrosides and mucopolysaccharides which seem essential to normal mental development in the infant. Lactose has a beneficial influence in changing the bacterial flora of the intestinal tract; it is reported also to aid in the assimilation of calcium and possibly magnesium. Babies fed diets fortified with lactose seem to develop firmer muscle structures than those fed diets supplemented with sucrose or other carbohydrates. Total weight gain is not the best criterion for judging the adequacy of a diet. One author classified lactose as a structural carbohydrate and most of the other sugars as energetic carbohydrates. If glucose can be

called a quick-energy sugar, it might well be that lactose could be called the sustained-energy sugar.

Lactose has enjoyed some acceptance as an ingredient in special diets, and some of the features just mentioned may apply equally well to such uses, even when adult or geriatric diets are involved. Some have claimed that diabetics can tolerate lactose levels far in excess of what might be expected, compared with sucrose or glucose. It may well be that the slow-burning characteristic of lactose helps in this respect. Some accepted diabetic ice creams have relatively large levels of lactose.

Food uses. In the food field there are many places where lactose can be used to advantage. Generally speaking, however, if the other constituents of milk can be utilized, too, it may be less expensive to use milk products rather than isolated, refined lactose. If, for instance, lactalbumin and milk minerals do not adversely affect the finished product, it may be that dry whey is the most economical source of lactose. If casein can also pay its way, the choice may be nonfat dry milk; and if butterfat also can be counted on to render value received for what it costs, the sources of lactose may be dry whole milk, or even fluid whole milk. In many applications, lactose per se is the choice of the food processor, just as he may well prefer to add butterfat to achieve some desired characteristic, rather than to use whole milk.

The wide variety of products produced by the baker affords a fertile field for lactose. Toppings and icings are often criticized for being too sweet. Replacing from 15 to 20% of the sucrose with lactose not only overcomes this objection but increases the color stability, if color is added, and enhances the flavor. Crême centers for cookies likewise can often be improved. Pie crusts can be made flakier and often improved in flavor by adding from 6 to 8% of lactose to the flour. Lactose has a sparing effect upon the shortening, as well as giving a better golden-brown color to the baked crust. Sogginess of the crust can be reduced by applying a lactose-solution wash to the surface before baking. This is especially helpful in the commercial production of pies, in which extended shelf-life is important. The same applies to meat pies and frozen pies of various kinds. Generally speaking, many of the pie fillings, either fruit or custard, can be improved by substituting lactose for a percentage of the sucrose.

In the candy industry, lactose contributes to the characteristics of many candy varieties. In the past, this has generally been achieved by adding milk, but with lactose available as an economical ingredient the candy craftsman has more leeway for expressing individuality. This is especially true in such traditional candies as the fudges and caramels. Not only is flavor often enhanced but lactose changes the crystallization characteristics of the sucrose and invert sugar, may give a better body or texture, or may improve chewiness and shelf-life. Recent work at the Southern Utilization Research and Development Division[1] has pointed up some of the advantages in the use of lactose in candy-making. There is much room for experimentation and research in this field.

There seems to be a place for the use of lactose in the processing of some fruits and vegetables. It will often enhance the flavor and give a better color. Frozen fruit juices are also possibilities.

Potato chips and French-fried potatoes can be given a deeper, more uniform golden color if they are dipped in a lactose solution before cooking in deep fat.

In the dairy industry finely powdered lactose has been used to seed sweetened condensed milk and thus improve the finished body or texture. Lactose has been used as an ingredient to improve cheese spreads. In the manufacture of chocolate drinks, the addition of a small percentage of lactose seems to enhance the flavor. Generally speaking, however, most of the dairy products are so shackled by laws, such as our system of issuing standards of identity, that there is little room for the possible use of lactose in their preparation, even though a nutritional or economic advantage may be demonstrated.

Lactose is a large component of dry coffee-cream preparations and it may be used to advantage in such items as dry soups etc. which are to be dispensed from vending machines.

It seems there is no limit to the variety of applications where lactose might be used. It would probably work if sprinkled on icy walks in place of sand and the chances are, when it dissolved, it would be less harmful to the lawn than the salt often used. We read of using lactose in the silvering of mirrors and in the manufacture of pyrotechnics, and some of you may have seen branches covered with rocks of lactose in a cordial or liqueur bottle prominently displayed for show purposes. A research worker in one of our leading dental schools came up with the idea of mixing large lactose crystals in varying proportions in the dough used in making small cookies. When checking to see how well a client's dentures fit, he would begin feeding the cookies with only a few lactose crystals in them and then work up the scale. The lower the level at which the client could detect the harmless, hard crystals, the better his dentures were seated.

Lactose is used in many different applications and the chances are that its uses will be extended further. Manufacturers are equipped to offer most any grade or variety which may be demanded. With improved methods of production, and corresponding reductions in cost, this segment of our dairy industry seems destined for further expansion and growth.

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[1] U. S. Dept. of Agriculture, New Orleans, Louisiana.

OUR INDUSTRY TODAY

FUTURE UTILIZATION OF DAIRY PRODUCTS[1, 2]

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The dairy industry of the United States, in 1956, had a sales volume for its products of slightly over 4.5 billion dollars. Two and one-half million farm families derived the major portion of their income from milk and over 30 thousand plants employed 250,000 workers to process and deliver the products manufactured by the dairy industry. The dairy products made available to American consumers furnished about 30% of the dietary protein and although per capita consumption of milk-equivalent now exceeds 700 lb., each year the industry is confronted with surpluses in excess of 5 billion pounds.

The 25 million dairy cows that produce the estimated 127 billion pounds of milk per year contribute, through dairy products, about 15% of all retail food sales. In addition to this, dairy cows account for approximately 40% of the beef and nearly all of the veal consumed in this country. These truly remarkable animals, because of their unique ability to convert crude forms of forage and grain to highly nutritious foods for humans, have attained an important position in our agricultural economy.

Although we do not wish to belabor the production aspects of milk, it is important to consider some of the present-day trends that might determine how much milk, in its various forms, will be available in the future for processing and distribution. Current statistics on a nation-wide basis are not readily available, but it is generally agreed that the number of dairy farms is steadily declining. In Pennsylvania, the agricultural economists tell us that between 1950 and 1955 the number of commercial dairy farms decreased 12.6%. In spite of this, milk production increased 18.4%, owing partly to increased size of remaining operations and partly to increased production per cow. Unfortunately, dairy farming still exists as a seven-day-a-week operation and is becoming less and less attractive to young people. Another deterring factor in the picture is that the capital investment required to establish a dairy farm is three times that of 1942. Even a modest 20-cow operation requires an investment of at least 30-40 thousand dollars for land, equipment, and cattle. The trend, therefore, appears to be the gradual expansion in size of the milk production unit, with greater emphasis on efficiency of production through semi-automation, improved forage, and greater production per cow.

The dairy industry has traditionally emphasized its butterfat production, with less attention being given to the other components of milk. Breeders of dairy cattle have attempted to increase the butterfat content of milk from both the high- and low-fat breeds at the expense of volume of milk production. There was justification for this practice when butter utilized from 50 to 75% of the milk produced, but with only 23% of the butterfat going to this commodity today, the market incentive is in favor of the milk containing from 3.8 to 4.0%. Because of this, some dairy geneticists are suggesting that the high butterfat breeds should select for milk yield rather than butterfat content. One group of workers has demonstrated a 1,000-lb. increase in milk production for each 0.2% genetic loss in butterfat content. The dairy manufacturing division of the industry today is favoring the 4.0% fat milk, so producers should now attempt to supply a product that lends itself most satisfactorily to current market demand.

The dairy industry historically has exhibited an attitude of ultraconservatism. Much of this, we believe, can be attributed to the conditions under which it has had to operate. Because of the dietary importance and inherent perishable nature of milk, strict state, municipal, and federal regulations have been established to control the production, processing, distribution, composition, and sanitary quality of all dairy foods. Moreover, in order to stabilize markets and insure an ample supply of dairy foods, federal and state milk-marketing orders have been established in most of the major milk markets of this country. These orders set the price paid to milk producers and, in some state programs, the price charged the consumer. Incentive for new product development has waned because of difficulties in having standards of identity established under existing state or federal food laws. In spite of this, the dairy industry has made substantial progress with its existing products and modifications thereof.

Although milk and dairy foods are unquestionably a permanent part of the American diet, the industry has too often rested its case wholly on the merit of its products and their current market acceptance. Increasingly aggressive competition from other segments of the food industry, as well as shifts in dietary

[1] Authorized for publication as Paper No. 2210 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] Paper presented at Symposium on Future Utilization of Agricultural Commodities, American Chemical Society Meeting, September 11, 1957, New York, N. Y.

food fads, emphasizes the fact that there will be little room for complacency in this or any other food industry in the future.

Milk and the commonly known products made from milk have long enjoyed the confidence of consumers, because of their taste appeal and nutritive value. In their familiar forms they are used in more or less predictable quantities, which change over the years, sometimes sharply, sometimes gradually.

The domestic use of butter dropped sharply during World War II because of rationing, and later because of the relaxing of restrictions on sales of oleomargarine. The total consumption loss amounted to about 7 lb. per person, equivalent to 140 lb. of milk or, for the country as a whole, approximately 22 billion pounds. Had butter consumption remained at the 1941 level, today there would be a shortage of milk rather than a surplus.

On the other hand, the per capita consumption of most other dairy products, particularly hard cheese, cottage cheese, fluid skimmilk, and dry skimmilk, has been on the increase. Unfortunately, the increased rate of consumption of these products, even with the increase in population, has not been able to overcome the result of lowered butter sales and the upward trend in milk production.

Another factor in the total picture is that increases in the use of nonfat products, coupled with sales of nonfat dry milk to the government, have encouraged manufacture of these products from fluid skimmilk, which at one time was wasted or fed to farm animals.

The result of these trends has been a continuing accumulation of surplus products in the hands of the government, under the support program. No immediate relief from this situation is in sight, but eventually population may catch up with milk production, as it will with the production of other foods, and today's problem in retrospect may be viewed as a most happy situation.

It is unlikely that the consumption of butter will change materially in the next several years although, because of the unique flavor qualities of butter and milk fat, it is doubtful whether the potentialities of this product have been fully realized.

The per capita consumption of fluid milk and cream, considered collectively, has been relatively stationary since 1948, except for the artificial stimulation furnished by the School Milk Program in the last few years. Cream consumption has been decreasing, but this has been offset by slightly greater milk consumption, particularly by the popularity of the product known as "half 'n' half," which contains about 10% fat and, at a lower cost, adequately substitutes for table cream.

The only significant increase in use of fresh dairy products has involved cottage cheese and fluid skimmilk. It is generally thought that interest in these products has been the result of the greater attention to diet calories and the recent adverse publicity on dietary fats; yet, the greater use of these foods has not significantly affected the per capita use of milk and cream, nor has the recent significant increase in home consumption of nonfat dry milk done so. The latter product, because of its low cost and easy dispersibility, is known to have been used by consumers for fluid milk purposes, yet seems to represent additional use rather than displacement use.

Much research is now in progress to improve the flavor and storage characteristics of dried milks. Should this prove successful, and should dry products having a fresh, fluid milk flavor on reconstitution be placed on the market at a lower price, it seems certain that the dry product will make substantial inroads into fluid milk sales. In fact, a high quality, beverage-grade, dry whole milk could offer serious competition to fresh fluid milk. Such a product would tend to encourage dairy farming in areas where milk can be produced most economically and efficiently. So far as the United States is concerned, these areas do not coincide with many of the current milk-producing sections close to large population centers. Moreover, the billions of dollars invested in urban fluid milk processing plants could become marginal if dry whole milk proves to be the ultimate consumer product. In this eventuality, it would be expected that distribution patterns and home practices with milk would undergo considerable change. Thus, the potentialities of dry whole milk must not be treated lightly.

It might be mentioned that lack of acceptance of dry milk, in present form, is quite largely a matter of conditioning and habit. People form food likes and habits as a result of what they have become accustomed to. So long as the familiar foods are of good quality and available, it is well-nigh impossible to convert them to something unfamiliar, even though the latter may be superior nutritionally. This psychological factor is one which all new foods and new varieties of foods must overcome, to establish themselves in the market. Naturally, the degree of difference, the price advantage, and convenience are factors in the acceptance of any new or different food product.

Dry whole milk, to be successful in domestic food channels, will have to be free of the astringent, chalky, and stale flavors currently characteristic of the product. The American consumer has established fresh, high-quality fluid milk as his flavor standard. Unless dry whole milk can achieve this beverage standard, it seems doubtful that it will ever enjoy an expanded market. A second, and perhaps equally important consideration, concerns ease of reconstitution in water. In this connection, the experience which has been gained with nonfat dry milk is most revealing. The improvement in its dispersibility through so-called instantizing processes unquestionably has been

a great impetus to consumer acceptance. Since this pattern has been set, it seems certain that dry whole milk must also be readily dispersible if it is to enjoy widest use.

Two innovations designed to furnish consumers with milk under reduced costs of distribution have been investigated and even tried commercially. One is the preparation and sale of three-to-one fresh concentrated milk. Owing to the removal of water, this product can be refrigerated and handled more cheaply than its fresh fluid counterpart. However, the American housewife did not show any sustained interest in the product, despite its greater potentialities in use and despite its space-saving character, except when the price was significantly lower than fresh fluid milk, which does not require adjustment with water. Frozen concentrated milk has been developed technically to the point where it could be sold as a frozen food. It has a relatively long keeping-period and could substantially reduce distribution costs, but the extra inconvenience to the consumer of defrosting and reconstituting it makes its acceptance so problematical that no one in the dairy industry is willing to risk the loss possible in an all-out effort to market it.

Evaporated milk has been a family staple for three generations. It has been the proverbial "cow in the pantry" and the most commonly used and generally satisfactory substitute for breast-milk in infant feeding. In recent years, a great deal of effort has been expended in seeking means by which the color can be lightened and the caramel-like flavor reduced. Here again, if a white canned milk with a fresh milk flavor were placed on the market at a lower price than fresh fluid milk, it seems axiomatic that many consumers would use it in place of the fluid product. Whether it would contribute to the consumption of more total milk is highly questionable. Then, too, it is likely that some present uses of evaporated milk are dependent on its color and flavor, which is of long standing. Consequently, any change, even though thought to be an improvement, may not be favored by all consumers. This might make it necessary or desirable to continue the manufacture of the present type of evaporated milk as well as an improved type.

Perhaps in no dairy product group has there been so general an increase of use as in cheese. Compared with 1934, 80% more cheese is being consumed by the average American. The reasons for this are not entirely clear, but certainly the improvement in quality, the manufacture of more varieties, the development of process cheeses and cheese spreads, and the use of packages, cartons, jars, and individual wraps have been important factors. Cheese smorgasbords are now the rule rather than the exception in every store and supermarket, and the appetizing displays of all sorts of cheese in convenient packages have certainly been educational and sales-provoking. The proliferating tendency in styles and varieties of cheese has been less subject to restriction by governmental regulations than is true of some other dairy products; this, too, is perhaps a factor in the increase of milk outlets in the form of this excellent protein food.

Dry milk of the nonfat variety has enjoyed increased utilization since the war, as an ingredient in other foods and feeds. Particularly has this been true in the baking industry, in meat products, in confections, and in prepared dry mixes for baking in the home. It seems unlikely that the greatest potential use of dry milk in human food products has been reached, but little increase has been noted in the last two or three years. It has been suggested that the lack of new-use research and development by the dry milk industry has been at least partly owing to the ease of reducing surplus production through sales to the government.

Although considerable increases in the consumption of nonfat and low-fat dairy products have occurred in the last decade or so, the increasing population has not significantly reduced the surplus of total milk, because of a comparable increase in production. Thus, the effects of the drastic drop in consumption of butter, during and following the war, have not been overcome; and the increased use of nonfat milk solids, while highly desirable in making better use of these nutritious food elements, has not greatly affected the surplus milk problem.

Thus, the question logically may be raised—can research and development do anything to alleviate this problem? The chances seem reasonable that they can, if attitudes and efforts of the various interested groups of the dairy field are adjusted accordingly. Until recently, milk has been considered mainly as an agricultural end-product. The idea has been to give milk mild processing, adequate for public-health purposes, and to convey it to the consumer with little change from its original state of synthesis by the cow. This line of thinking also has pervaded manufacture of most dairy products. It has made for relatively inflexible use of milk, and has created substantial problems in certain dairy products. The problems of flavor and color in evaporated milk, and flavor and dispersibility in dry whole milk, may stem largely from insistence that milk for these products be used in essentially unaltered form. As mentioned previously, local state and federal regulations, as well as attitudes of the consuming public, have tended to confine milk processing along rather narrow lines. Appropriate safeguards in the public's interest are always in order and are needed particularly during such times as when proper sanitation in an industry is to be established. Unfortunately, the welter of customs, traditions, and regulations which bear on the dairy industry has had an inhibiting and demoralizing effect on product

research and development efforts. It now seems clear that future progress will depend on the consideration of milk as a flexible raw material, available in tonnage quantities, and viewed in the light of modern science and technology.

Greater emphasis is needed on custom manufacture of milk components, such as fat, protein, and lactose, for various special purposes. If such processing can be accomplished at reasonable cost, it should enable development of new products, as well as improvement in the long-established traditional dairy products. In this connection, a high speed clarifier-separator capable of continuous fractionation of milk into its major components would be most useful. Fat might be separated as an oil or high-testing cream; protein might be removed by sedimentation and continuous de-sludging, and lactose obtained as a solution, along with other milk solubles.

An appraisal of some of the potentialities of the major milk components seems in order:

Milk fat. At present, the assumption seems to be widely held that the same form of milk fat can be used satisfactorily in any dairy product. This is by no means the case. Some products are stored under refrigeration and are consumed quickly. Others are stored at room temperature and may be held for months prior to consumption. Some are rigorously heated, whereas others receive only mild heat treatment. Thus, it seems obvious that further improvement in dairy products is, at least in some instances, going to require special processing of milk fat.

A further point in this connection concerns flavor in milk fat. If there is anything distinctive about milk fat, it is its potential for developing various desirable types of flavor. There are many ways of enhancing and modifying the flavor of milk fat, including partial hydrolysis to yield cheese-type flavor, heating to intensify butter flavor, and heating with sugars to develop butterscotch flavor.

Until now, the assumption has been that all food applications requiring milk fat can be fulfilled either with butter or with cream. There is good reason to believe that this contented outlook has sacrificed many worth-while outlets to tailor-made products of the vegetable fat industry. The most popular chocolate bar in America derives its distinctive flavor largely from hydrolyzed milk fat. Cooked vegetables are at their best when seasoned with the flavor of heated milk fats. It is obvious that the application of milk fat for improving the flavor and texture of many types of foods has not received nearly enough developmental effort.

Milk protein. Good-quality dietary protein is needed throughout the world. Milk protein can fulfill this need and is doing so to some extent in the form of various milk products. However, essentially pure milk protein preparations with long shelf-life and available at reasonable price could be used to enhance the nutritive value of other foods and to improve conventional dairy products. One of the current difficulties in a number of stored dairy products stems from the browning reaction, which leads to stale flavors and other defects. This affords an additional reason for separate processing of milk protein and the reducing sugar (lactose) fractions of milk, which are the browning reactants.

Lactose. Perhaps the greatest challenge and the greatest potential for utilization of milk lies in lactose, milk's major constituent. Some ten billion pounds of whey, by-product of cheese manufacture, is largely wasted and, in fact, constitutes a major disposal problem for the dairy industry. This problem actually has two facets: not only is there no profit on whey but frequently high expense is involved in providing sanitary waste disposal for it in accordance with local health regulations. Thus, there is strong incentive for research on this problem.

Lactose represents 80-85% of the solids in cheese whey. Unfortunately, few distinctive uses for lactose have come to light which cannot be fulfilled as efficiently and economically by other carbohydrate sources. However, it is notable that no all-out effort on this problem has been made and, as an example of one type of use which could be exploited, the following developmental effort at our laboratory may be cited:

Whey is used to some extent as an animal feed, but because of its high lactose and low nitrogen content it has distinct limitations for this purpose. To overcome this difficulty, a process has been developed to completely ferment the lactose of whey to lactic acid and to use the lactic acid as a means of binding anhydrous ammonia in the product. The fermentation and ammonia addition are controlled automatically and the finished product can be concentrated to any desired level of total solids. This process substantially increases the nitrogen content of whey. In addition to the normal mineral and vitamin constituents, this product contains a harvest of bacterial cells and large quantities of ammonium lactate. Since ruminants can utilize simple forms of nitrogen, the product seems appropriate for their feeding. Preliminary observations indicate that a concentrated ammoniated whey product is nontoxic, can support growth in young dairy animals and milk production in adult cows. However, some problems of palatability are present. Products of this type warrant investigation for feeding other types of livestock and poultry, and for use as fertilizer.

It seems notable that no great effort has been spent on developing detergents, plastics, synthetic fibers, stabilizers, thickening agents, etc., from lactose, in the manner which has been so effective for other carbohydrate materials.

Summary

Dairy products undoubtedly will continue to hold a prominent place in the American diet, but there will have to be more diligent efforts to adapt them to changing market demands. The deep-seated food habits of American consumers are not likely to be altered greatly by any new dairy product unless it exhibits unique flavor qualities or applications to other processed foods. The dairy industry must prepare to meet its competition in the domestic foods field with products, rather than depend upon historical tradition to carry it along.

We are not obsessed with the idea that consumers can or should greatly expand their per capita intake of dairy foods. In this land of abundance and variety in foods, it is only natural that consumer selections will be made on the basis of taste appeal, nutritional quality, availability, and price. Dietary trends that relate to the physiological demands of our changing way of life may also dictate the future food patterns of our population. In milk, however, we have a raw material which contains most of the known nutritional requirements of man and which, because of its dynamic state, can be converted into a wide variety of wholesome, taste-appealing food products. The outlook for milk is bright and its benefits to humanity can be broadened still further through an aggressive research and development program. It will be the job of the chemist and food technologist to explore more fully the potential food and industrial applications of this important agricultural raw material.

ABSTRACTS OF PAPERS

PRESENTED AT THE EASTERN DIVISION MEETING

SPRINGFIELD, MASSACHUSETTS, SEPTEMBER 16, 1957

Single versus duplicate determinations for the estimation of vitamin A and tocopherol in calf livers. MARTHA W. DICKS, J. E. ROUSSEAU, JR., H. D. EATON, R. TEICHMAN, AND H. L. LUCAS, JR., Storrs (Conn.) Agr. Expt. Sta. and North Carolina State College (Raleigh).

Data from eight randomized-block experiments, in which calves were fed fixed levels of vitamin A and/or carotene and/or tocopherol, were used to determine the effect on the error mean squares (block times treatment) of using the first duplicate determination for vitamin A or tocopherol concentration in the liver as compared to using the average of the duplicates. In general, the error mean squares of the average of the duplicates were slightly smaller in magnitude than those of the first duplicate; however, these reductions were found not to be statistically significant. The direct computation of the error mean squares was undertaken in contrast to the components of variance approach, because duplicates were run side by side in contrast to random order. Individual analyses of the concentration in the calves' livers exhibited only inappreciable random variation when using the first duplicate as compared to using the average of the duplicates. For purposes of laboratory control, standard deviations of a single determination for liver vitamin A or liver tocopherol were derived, and control charts were constructed from which the reliability of a group of sample determinations could be evaluated.

Effect of feeding various levels of 2,6-di-tertiarybutyl-4-methyl phenol to lactating dairy cows.[1] R. TEICHMAN, M. E. MORGAN, PATRICIA MACLEOD, AND H. D. EATON. Storrs (Conn.) Agr. Expt. Sta., Storrs, Conn.

In a recent study conducted at this Station by De Luca *et al.*, 1956, 2,6-di-tertiarybutyl-4-methyl phenol (BHT), when fed at a daily rate of 0.01% of ration, was found to be ineffective in reducing the incidence of copper-induced oxidized milk flavor. It was recognized that higher intakes of this antioxidant might be effective in reducing the incidence of copper-induced oxidized milk flavor, and this study was initiated to explore the possible effectiveness of higher levels of BHT. Two separate trials, each with 16 cows selected for producing milk which would exhibit oxidized flavor 72 hr. after the addition of 5.0 p.p.m. of copper, were conducted in February and in November, 1956. Following a 1-wk. standardizing period, they were fed daily intakes of BHT, 0.00, 0.01, 0.03 and 0.09% of the ration (90% dry matter basis) for a 4-wk. comparison period. BHT fed at the 0.03 and 0.09% levels significantly reduced, but did not completely inhibit, the incidence of copper-induced oxidized milk flavor. BHT was transferred to the milk in increasing concentration with increased intake.

Effects of various postpartum treatments upon the reproductive efficiency of dairy cows. N. DE D. BROWN, JR., K. O. PFAU, R. E. MATHER, AND J. W. BARTLETT, New Jersey Agr. Expt. Sta., Sussex.

All Holstein cows in the herd of the Dairy Research Farm, Sussex, New Jersey, were assigned as they freshened, either to Group A, normal calving, or to Group B, abnormal calving (dystocia and/or placenta retained more than 12 hr.) Cows were assigned alternately to treatment lots as they calved: Group A, Lot I, no treatment; Lot II, one intrauterine treatment of Aureomycin (500 mg. oblet) within a day after parturition. Group B, Lot I received Sulmet (sulfamethazine—15 g. per treatment) and Lot II received Aureomycin (500 mg. per treatment) until cured. The numbers of cows in the four lots were 92, 80, 24, and 26, respectively. Services per conception were 1.42, 1.74, 1.96, and 1.62; per cent nonreturn to first service, 67, 54, 46, and 62, and days from calving to conception, 97, 102, 118, and 101, respectively. Differences due to routine Aureomycin treatment of normally freshening cows did not approach significance, but certainly treatment was not beneficial. Cows freshening abnormally responded more favorably to Aureomycin than to Sulmet (P ca. 0.09 for conception interval). Breeding efficiency of cows calving abnormally and treated with Aureomycin approached that of cows calving normally.

Comparison of chromium ratio and lignin ratio techniques for determination of digestibility of hays. J. G. ARCHIBALD, D. F. OWEN, JR., H. FENNER, AND H. D. BARNES, Massachusetts Agr. Expt. Sta., Amherst.

In connection with two winter seasons' feeding and digestion trials with 16 cows, on the value for milk production of two common types of hay (legume-grass *vs.* grass alone), a comparison was made of the chromium oxide ratio

[1] This study was in part supported by grants from the American Cyanamid Co., Chas. M. Cox Co., and the Chas. H. Hood Dairy Foundation. The technical assistance of G. R. Farrington and R. M. Prouty was greatly appreciated.

and lignin ratio techniques for determining digestibility of the hays.

Digestion coefficients, when summarized for dry matter, protein, energy, crude fiber, and N-free extract from 16 individual trials, show that the chromium oxide method gave generally higher results (average 1.4 points) than the lignin method, with somewhat more uniformity, as indicated by generally lower standard errors of the mean values ($\pm$ 1.1 *cf.* $\pm$ 1.8).

It is concluded that if the ratio technique system of determining digestibility of forages is employed, the chromium trioxide method is to be preferred to the lignin method, because of more consistent results and greater ease of determination of chromium in feces.

Influence of herd and stable-mate averages on production records of artificially sired (A.I.) progeny. S. N. GAUNT AND M. A. GOODWIN BARTLETT, Massachusetts Agr. Expt. Sta., Amherst.

As part of a study to determine the relative accuracy of various measures of a dairy sire's breeding value, the effect of the herd and four stable-mate averages on A.I. production records was determined. For this determination, 22,621 lactation records made in 1952–55 in 237 herds in New England and New York and their annual herd averages were used. This included 6,178 and 2,012 progeny records of 302 Holstein and 151 Guernsey A.I. sires, respectively.

Since herd, season, and year influences account for nearly half the variation in production records, adjustments for them should improve estimates of a dairy sire's breeding merit. Values for these adjustments were obtained by computing the intra-sire regression of the daughter's record on actual herd, and stable-mate, averages for lactation, 6-mo. season, running season (7 mo. average), and actual age. The average intra-sire regressions for milk and fat for each of the contemporary averages were, respectively: for Holsteins, 0.94, 0.95, 0.90, 0.98, and 0.89, and for Guernseys 0.89, 0.90, 0.96, 0.94, and 0.84. The differences among the individual regressions for Holstein and Guernsey milk were highly significant statistically and for Holstein fat significant.

Development of flavors in sterile milk by pure cultures of bacteria at low temperatures. D. S. ERICKSON, AND D. A. EVANS, University of Massachusetts, Amherst.

In this study, separate samples of sterile whole milk were inoculated individually with pure cultures of *Aerobacter aerogenes, Escherichia coli, Alcaligenes metalcaligenes, Corynebacterium simplex, Pseudomonas geniculata, Micrococcus fruendenreichii, Flavorbacterium arborescens,* and *Achromobacter superficiale,* using respective initial concentrations of 10,000, 50,000, and 200,000 organisms per ml. The inoculated samples were held at 5, 10, 15, and 20° C. Flavor examinations were made every 24 hr., and both standard plate and facultative psychrophilic plate counts were made when off-flavors were initially observed.

Results revealed temperature was more important than initial concentration of organisms in determining rapidity of off-flavor development. The rate of flavor production progressively increased as temperature increased from 5 to 20° C. All organisms studied produced definite off-flavors, irrespective of initial population or temperature of holding. Among the flavors observed were unclean, bitter, fruity, acid, nutty, and cheesy. The bacterial population at the time of initial flavor development ranged from 16,000 to more than 30,000,000 per ml. as determined by the facultative psychrophilic count. Standard plate counts, with but three exceptions, did not correlate well with corresponding facultative psychrophilic counts, and in many cases the standard plate incubation temperature completely inhibited growth of certain organisms.

Bulk farm tanks in Massachusetts—A survey of the current situation. MARCEL DANEAU AND JOHN H. BRAGG, University of Massachusetts, Amherst.

According to calibration records of the Massachusetts Division of Standards, as of April 1, 1957, there were 925 bulk farm tanks in the Commonwealth ranging in size from less than 100 to over 1,100 gal. Average capacity of these tanks is 295 gal. and the mode is 245 gal. These records also show that a larger proportion of tanks installed since 1955 have capacities of 600 gal. or more than of those installed in 1955. This indicates that the large farms were not necessarily the first to shift to bulk.

There is a definite relationship between the location of farms with bulk tanks and farms with 15 cows or more. In most instances, the areas with the most intense cow population had the greatest number of bulk farm tanks. Distance and location of market do not appear to have any bearing on the concentration of tanks. Two trends best describe the rate of installations: From 1952 until the spring of 1955, tank installations trended upward. Each year since then, the trend has been downward. From 1952 through April, 1957, there was a definite seasonal pattern to the installation of tanks with two high points, in the early spring and at the year-end.

Acid degree of milk obtained with pipeline and bucket milkers. R. G. JENSEN, A. C. SMITH, PATRICIA MACLEOD, AND L. R. DOWD, University of Connecticut, Storrs.

The purpose of this study was to determine how much properly operated pipeline milkers contributed to the acid degree value of milk. Samples of pipeline milk were compared to bucket-milked samples. Samples were taken from the milk of 17 and ten cows on two pipelines. Twelve sets of samples were collected in July, 1956, and 24 in December, 1956. It was found that pipeline milking as compared

to bucket milking increased the acid degree value (silica gel method) of milk held after milking for 72 hr. at 4° C., even though the pipeline had no risers and no observable air leaks. Pipeline treatment of winter milk ($P < 0.001$) increased the acid degree value over bucket treatment to a greater extent than did tratment of summer milk ($P < 0.05$). The average acid degree values for the samples collected in December were 0.76 for bucket milk and 1.31 for pipeline milk; for July samples, 0.61 for bucket milk and 0.87 for pipeline milk. Passage of winter milk through an intermittently operated diaphragm pump significantly ($P < 0.01$) increased the acid degree value of both the pipeline and the bucket-treated milk samples, when the milk was obtained from a herd in which five of 17 cows had been milked 10 mo. or longer.

PRESENTED AT THE SOUTHERN DIVISION MEETING

LITTLE ROCK, ARKANSAS, FEBRUARY 3-5, 1958

A study of the relationship between A.O.A.C., Mojonnier, and Watson Lactometer determinations of total solids in raw whole milk. N. R. THOMPSON, W. K. STONE, AND G. C. GRAF, Virginia Agricultural Experiment Station, Blacksburg.

Samples of single milkings from three to eight Holstein cows were combined to make a total of 159 one-day composite samples. Determinations of specific gravity were made with the large pattern Watson lactometer, and butterfat percentages were obtained by the Babcock method. The lactometer readings and butterfat percentages were used to estimate per cent of total solids in each sample. Determinations of total solids also were made by the Mojonnier procedure, by forced-draft drying oven, and by the official A.O.A.C. method. Percentages of total solids by the Watson lactometer method differed from the values obtained by other methods; ranging from 0.07% TS higher in the first month of lactation, down to 0.33% TS lower in the ninth month.

Influence of feed upon the composition of milk. I. I. PETERS, R. R. HARRIS, AND C. A. MULAY, Texas Agricultural Experiment Station, College Station.

The following information was obtained during a 12 wk., double-reversal feeding trial with two lots of ten cows each, six Holsteins and four Jerseys.

The estimated net energy and the protein content of the three rations were the same. Ration B (control) with no added fat contained 63% more crude fiber than Rations CT and CM. Ration CT contained 6% stabilized prime tallow; whereas, Ration CM contained 6% Marcol B-75, a commercial product consisting primarily of the methyl esters of cottonseed "foots."

The following differences were found to be statistically significant; With the Holstein group, the per cent lactose was higher from Ration B than from Ration CM, and per cent S.N.F. was higher from Ration B than from Ration CT.

With the Jersey group, the per cent fat was higher from Ration CT than from Ration B, and the difference (S.N.F.-lactose) was greater from Ration B than from Ration CT.

Both breeds showed higher, though not significantly, freezing points on the CT and CM rations than when on the B ration.

Effect of the dairy herd ration upon the susceptibility of milk to induced hydrolytic rancidity. W. B. GILMORE, J. J. WILLINGHAM, AND K. L. NEELEY, Texas Technological College, Lubbock.

Studies were made to determine the degree of suceptibility of milk to induced rancidity as influenced by the ration. The tests were made over a 1-yr. period at monthly intervals. With each sampling period, records were made concerning the nature of the ration. To measure the degree of hydrolytic rancidity, tests were made to determine the amount of free fatty acids in each sample. Tests for butterfat, chloride content, titratable acidity, pH, and free fatty acids were made on the fresh sample of raw milk. A portion of this same milk was treated by a method known to induce rancidity.

After 48 hr., both treated and untreated samples were tested for titratable acidity, pH, and free fatty acids.

Variations in the suceptibility of milk to induced rancidity were found to be closely related to the type of ration being fed.

Effect of various factors on the incidence and intensity of rancid flavor in raw milk handled in bulk tanks. R. L. VON GUNTEN, P. E. JOHNSON, AND H. C. OLSON, Oklahoma State University, Stillwater.

A survey of the Central Oklahoma milkshed was conducted to determine the incidence and intensity of rancid flavor in raw milk handled in bulk tanks. This study involved 249 milking and bulk-cooling systems.

Samples were taken from each farm tank on an every-other-day basis as the milk was collected. After the samples were held at 45° F. for two days, chemical analyses showed that approximately 10% of the samples had high acid degree values in the range indicative of rancidity.

The survey included 221 open top–type bulk tanks and 28 vacuum–type tanks. There was a high incidence of rancidity in milk from the vacuum tanks, but laboratory experiments failed to show that the vacuum was a factor in increasing lipase activity. Except for the vacuum-type tank, there was little difference in the incidence of rancidity in the milk handled in the various brands of bulk tanks. It appeared that pipelines, with either vacuum releasers, milk pumps, or vacuum tank assemblies are the most important factors to consider as causes of rancidity.

The influence of rapid cooling on the development of a rancid flavor in milk produced by bulk systems. P. E. JOHNSON AND R. L. VON GUNTEN, Oklahoma State University, Stillwater.

The influence of rapid cooling of milk on the activation of lipase was suspected while studying other phases of the rancid-flavor problem in bulk-handling systems.

Samples of milk from individual cows were collected after passing through a pipeline system, and were immediately divided into two portions. One portion was cooled immediately, whereas cooling was delayed on the other portion. The samples were held for 48 hr. before fat acid values were determined. Highly significant differences occurred in the fat acid values of the two portions of the split samples. Average increases in the fat acid values of the rapidly cooled samples over the slowly cooled samples averaged from 0.67 to 1.42.

A delay of 30 min. after the completion of milking, before turning on the refrigeration in the bulk tank, was also effective in lowering fat acid values.

Growth rate and forage intake of young dairy calves on pasture, with and without grain supplementation. J. W. RUST, L. D. BROWN, D. M. SEATH, AND J. H. DRUDGE, University of Kentucky, Lexington.

Sixteen 3½ to 4½ mo. old calves on good bluegrass pasture were divided into two comparable groups based on breed and body weight. One group of calves receiving 3 lb. of grain per day gained an average of 1.2 lb. per day throughout the summer; whereas, the other group of calves receiving no grain gained an average of 1.0 lb. per day. The average increase in height at withers was 6.5 and 5.7 in. and the average increase in heart girth was 11.5 and 10.5 in. for the grain and no-grain groups, respectively.

Dry matter intake and digestibility were determined by the chromogen chromic oxide techniques. The calves receiving grain had an average total dry matter intake of 3.6 lb. per day for each 100 lb. body weight. Average dry matter intake from pasture alone was 2.6 lb. per 100 lb. body weight for the group receiving grain, as compared to 3.7 lb. for the group receiving no grain. Similarly, the average dry matter digestibility coefficients were 65.6 and 64.5%, respectively.

Periodical fecal examinations were made for the presence of parasite eggs. In general, the grain-fed calves had a lower level of parasite infection than the no-grain group.

A preliminary analysis: Birthweights of Holstein and Jersey female calves born in southern Louisiana. E. J. STONE, E. B. MORGAN, J. E. JOHNSTON, AND J. B. FRYE, JR., Louisiana State University, Baton Rouge.

Variation in birthweight of calves and its effect on the subsequent growth and productivity of dairy cows is of particular concern in the Deep South. This is a preliminary report on the birthweights of calves of the Holstein and Jersey breeds born under Louisiana conditions. Birthweight data on single births of 129 Holstein and 87 Jersey female calves for the period 1951 through 1957 have been studied. A statistical analysis including birthweights by breeds, sires, years, months, and seasons has been made. Differences in birthweights among months for the Holstein calves were significant at the 5% level of probability. Among years and among months, differences in birthweights for Jersey females were highly significant (1% level). When the data were analyzed by age of the dam at calving for both breeds, no significant differences were shown either among years or among months. Correlation coefficients between birthweights and age (using all births when age of dams was limited from 24 to 84 mo.) were highly significant (1% level) for both breeds.

Relationship between estrogenic potency of soybean and cottonseed meals and lactation response. G. E. HAWKINS AND K. M. AUTREY, Alabama Polytechnic Institute, Auburn.

This study was originated to compare the lactating qualities of feeds with different estrogenic potency.

The 25 experimental cows were divided into five similar groups. Ration variables and percentage of TDN intake supplied by each were: A, alfalfa hay (72) and concentrate (28); B, alfalfa hay (71) and cottonseed meal (29); C, alfalfa hay (74) and soybean meal (26); D, Johnson grass hay (68) and cottonseed meal (32), and E, Johnson grass hay (64) and soybean meal (36). There was no increase in uteri weight of mice when fed alcoholic extracts from the concentrate mixture, soybean meal, alfalfa hay, or Johnson grass hay. Alcoholic extracts of cottonseed meal supplied estrogenic activity equal to 0.0042 μg. of diethylstilbestrol per g. of feed. It is surprising, therefore, that the concentrate mixture which included 40% cottonseed meal did not show estrogenic activity. Persistency of milk yield in per cent

by dietary groups was: A, 103; B, 92; C, 92; D, 87, and E, 84. Nutrients from cottonseed meal, the feed that showed the greatest estrogenic activity by the mice uteri assay, were similar to nutrients from soybean meal and somewhat inferior to nutrients from a concentrate mixture, for milk production.

Estrogenic potency of freshly cut forages in relation to their value as sources of nutrients for milking cows. G. E. HAWKINS AND K. M. AUTREY, Alabama Polytechnic Institute, Auburn.

This experiment to determine the relationship between estrogenic potency of the forage and milk production involved 15 dairy cows. The experimental rations were. A, alfalfa hay; B, green alfalfa plus alfalfa hay; C, green oat forage plus alfalfa hay; D, green crimson clover plus alfalfa hay, and E, green subterranean clover plus alfalfa hay. Cows on Rations B, C, D, and E received 50% as much hay as they ate before the experiment, plus 50 lb. of the green forage. In addition, each cow in all groups was fed 4 lb. of ground shelled corn daily.

Estrogenic potency of the forages was measured by the mouse uterus assay by adding alcoholic extracts of the forages to a basal diet with low estrogenic potency. Weights of uteri from mice fed the experimental forages were: A, 6.1; B, 11.5; C, 3.6; D, 8.9, and E, 12.1 mg. Uteri weights per g. from mice fed the basal diet, and basal diet plus 0.04 μg. of diethylstilbestrol, were 3.5 and 22.1 mg., respectively.

Persistency of milk production in per cent on the five rations was: A, 100; B, 116; C, 121; D, 105, and E, 103. There was no consistent relationship between the estrogenic potency of the forages and the persistency of milk yield.

Comparative study of the effects of intravenous and nasal administration of the let-down hormone on residual milk. HIROO YAMAMOTO, Loyola University of the South, New Orleans, and CHARLES W. TURNER, University of Missouri, Columbia.

Ten, 20, and 30 units of the Armour oxytocin intrajugularly administered to five sterile heifers with experimentally developed mammary glands, resulted in a mean (four trials) per milking let-down response of 13.1, 12.1, and 15.5%, respectively, when the heifers were remilked from 1 to 2 min. The mean residual milk yield per milking of six trials without the exogenous let-down factor was 4.8%.

The mean response with 600 units of the bovine posterior pituitary extract introduced nasally at 15, 30, and 45 min. for two trials per interval for six treatment days gave additional yields of 6.2, 11.1, and 9.5% at the respective intervals. The extract in 800 units at the same intervals and trials stimulated the removal of 7.1, 8.0, and 10.0% of residual milk.

These data indicated that high dosages of the order of 20-30 times the effective intrajugular units were necessary in order to elicit reasonably comparable let-down response through nasal application.

The growth of Jersey and Sindhi-Jersey crossbred heifers at the Iberia Livestock Station. J. L. FLETCHER, S. L. CATHCART, AND C. E. HYDE, USDA, ARS, Dairy Cattle Research Branch and Louisiana Agricultural Experiment Station, Jeanerette.

In the Red Sindhi–Jersey crossbreeding work at the Iberia Station, monthly weights of the heifers were recorded from birth to one year. The crossbred heifers included F_1, F_2, ¼ Sindhi, and ¾ Sindhi combinations. The crossbred heifers were significantly heaiver than the Jersey heifers at birth, at 6 mo., and at 12 mo. of age. The weights at birth, 6, and 12 mo. were analyzed for possible effect of season of birth. Only at 12 mo. of age was season found significantly to affect the weights.

Of considerable interest was a comparison of the growth curve of heifers at this station, to standard growth curves reported at other stations. At birth, the weight of the Jersey heifers was practically identical with that used in the Missouri standard, whereas the F_1 crossbreds exceeded this standard. At 3 mo. of age, both of these groups surpassed the standard. The F_1 heifers still were above the standard at 6 mo., but the Jerseys were slightly below. Between 6 and 12 mo., both Jersey and crossbred heifers fell behind the standard curve and at 12 mo. the Jersey heifers were more than 100 lb. below the standard.

Practical methods of keeping bulls cool during the summer months. T. E. PATRICK, H. C. KELLGREN, J. E. JOHNSTON, AND C. BRANTON, Louisiana State University, Baton Rouge.

Studies made during recent years show a sharp decline in semen quality and fertility of both dairy and beef bulls during the late summer and early fall. Records at the Louisiana Station reveal that more than one-third of the bulls investigated showed partial or total sterility during the months of August and September. This study was initiated in an effort to find a practical method of keeping bulls cool, so as to maintain a high level of fertility throughout the year.

Twenty-five bulls representing the Jersey, Guernsey, and Holstein breeds were assigned to five groups. Group 1 was housed in box stalls in a pole-type barn open on two sides. Group II and III were housed in a large barn equipped with ten overhead fans and two exhaust fans. In addition, Group II was exposed to a very fine mist-type water spray.

Groups IV and V were kept in individual paddocks which were equipped with aluminum-covered sheds open on one side. The Group IV paddocks also had natural shade.

Preliminary observations indicated that the relative comfort of the bulls in descending order was Groups II, III, IV, V, and I. Semen quality and fertility data will be presented.

Homologous vs. nonhomologous nutrient systems for the culture of bovine testicular tissues. M. A. BROWN, Texas Agricultural Experiment Station, College Station.

In the intact organism, nutrition is furnished entirely by a homologous system. Numerous workers have found, however, that nonhomologous nutrient media more adequately support growth and maintenance of tissues and organs in vitro. Apparently, processes for preparing nutrient media and for culturing tissues alter either the characteristics of the media or of the tissues, so that the latter are not adequately nourished by homologous fluids.

In a tissue culture study conducted at this station, it was found that a nutrient fluid containing bovine serum, bovine embryo extract, and a balanced salt solution (homologous nutrient system) sustained adequate growth of cultures of bovine testicular tissue in vitro an average of 38 days per culture. Nonhomologous nutrient systems containing chick embryo extract, balanced salt solution, and bovine serum, horse serum, fowl serum, human cord serum, or human ascitic fluid maintained similar tissues in culture for an average of 33, 23, five, eight, and four days, respectively. This may indicate that homologous nutrient systems may be used successfully to nourish cultures if optimum culture techniques are employed.

Some observations on reproductive performances of dairy cattle in Louisiana. J. G. HALL, C. BRANTON, E. J. STONE, R. B. LANK, AND J. B. FRYE, JR., Louisiana State University, Baton Rouge.

Observations were made to determine duration of estrus, length of estrous cycles, incidence of anestrus, and other related factors affecting breeding efficiency. Observations for heat were made at 6-hr. intervals, except for four, ten-day seasonal periods, when observations were made at 2-hr. intervals. Time of ovulation was determined by rectal palpation at 6-hr. intervals after the end of estrus.

Preliminary results on 219 cows and heifers, involving 837 heat periods, gave an average of 12.56 ± .43 hour for the duration of heat for the observations made at 6-hr. intervals. Observations made during three, 10-day periods in January, August, and September, involving 124 heat periods, gave an average duration of heat of 11.47 ± .48 hour. The duration of standing estrus was 10.58 ± .20 hour, with 10.04% not-standing.

The average length for 615 estrous cycles was 20.67 ± .49 day, with 21 days the modal class.

The efficiency of observations for estrus was 85.41%, when the 0600 and 1800 hr. observations were considered. However, efficiency was 94.02% when the 0600, 1200, and 1800 hr. observations were used.

The incidence of postpregnancy estrus was 2.22%. These estrous periods were not included in the 837 periods used to estimate average duration of estrus.

A study of factors affecting the number of cows bred artificially. DURWARD OLDS, ELLIS MULLINS, AND D. M. SEATH, University of Kentucky, Lexington.

Fifteen southern states bred artificially an average of 10.2% of their dairy cattle during 1956, compared to a national average of 25%. Attempts have been made to explain this contrast on the basis of lower concentration of cows, fewer telephones, and poorer roads in many areas of the South. The present study was designed to determine the relationship between the number of cows bred artificially per square mile in the 48 states and the following three factors: *(1)* number of dairy cows per square mile, *(2)* number of rural telephones per square mile, and *(3)* the percentage of farms on unimproved dirt roads.

The multiple correlation coefficient was .65, indicating that 42% of the variation in number of cows bred was owing to the combined effects of these three factors. The correlation coefficient with cows bred was .51 for cow population, .38 for number of rural telephones, and —.55 for percentage of farms on dirt roads.

Multiple regression calculations revealed that seven states (Alabama, Kentucky, Louisiana, Maryland, Mississippi, Tennessee, and Virginia) bred fewer cows artificially than would be expected; whereas, eight states (Arkansas, Florida, Georgia, North Carolina, Oklahoma, South Carolina, Texas, and West Virginia) bred more cows artificially than would be expected.

The manufacture of Cheddar cheese from pasteurized homogenized milk. I. I. PETERS, C. A. MULAY, AND G. L. DALTON, Texas Agricultural Experiment Station, College Station.

The following information was obtained on four trials on Cheddar cheese made from raw, pasteurized, and pasteurized, homogenized milk, subjected to 1,000 and 2,000 p.s.i., respectively.

Milk in each trial was of similar composition and quality.

Average pounds of cheese obtained per 100 lb. of milk were 10.75 from raw, 10.75 from pasteurized, and 11.19 and 10.75 from

pasteurized, homogenized, at 1,000 and 2,000 p.s.i., respectively. Average calculated yields of cheese based on 39% moisture, listed in the above order, were 11.36, 11.45, 11.77, and 11.77 lb.

The higher possible yield from pasteurized over raw milk was owing to greater S.N.F. utilization; whereas, the higher possible yield for pasteurized, homogenized milk over the raw milk was the result of greater fat utilization.

Intermittent weighing of cheese during drying showed a decreasing drying rate from raw to pasteurized to pasteurized, homogenized milk cheese.

A study of selected chemical and physical properties of high solids cultured milks. JOHN JAYNES, E. R. JARMAN, AND J. J. WILLINGHAM, Texas Technological College, Lubbock.

The research work was designed to study several selected chemical and physical properties of high solids cultured milks.

The level of solids of the three groups of milk studied was 16, 18, and 20% total milk solids. All samples of each group contained 1% butterfat.

Studies were made on these enriched milks to determine the effect of these high levels of nonfat milk solids on viscosity, pH, titratable acidity, and total volatile acidity.

Observations concerning the effect of added citrates were made, along with taste evaluations. Several trials involving homogenization of cultured milks and the effect of viscosity and wheying-off were made.

Influence of frequency of transfer and of incubation period on the activity of lactic cultures. H. C. OLSON, Oklahoma State University, Stillwater.

Five cultures—four active, and one slow due to bacteriophage infection—were transferred five times a week, twice a week, once a week in milk with calcium carbonate added and not transferred. The latter set of cultures was ripened in milk containing calcium carbonate and then held at from 40 to 50° F. At weekly intervals, each set was transferred and activity tests run on the ripened cultures after from 12 to 14 hr. of incubation at 72° F. After several weeks, it appeared that the frequency of transfer had no significant influence on the activity of the active cultures. The slow culture gradually increased in activity, except that it remained slow when stored in the carbonate milk without transferring.

Ten cultures, representing active and slow ones, were incubated for 16 hr. and for 24 hr. Activity tests, run at weekly intervals, indicated that the 24-hr. incubation was somewhat detrimental to the active cultures, but appeared to be beneficial to the slow ones.

Procedure for evaluating cottage cheese-type nonfat dry milk. W. K. STONE AND P. M. LARGE, Virginia Agricultural Experiment Station, Blacksburg.

To compare the curd-making property of a powder with that of a low-heat control, cottage curd is made from each dry product in small duplicate vats by a rapid short-set method. Twenty-five per cent low-heat starter containing 11% solids, made from the powder being tested, is added to each vat of 11% cheese-milk. The temperature is adjusted to 90° F. and rennet is added. When the pH of the curd is 4.70, it is cut into ¾-in. cubes, and the whey is slowly heated to 98° F. This temperature is maintained until the pH of the curd is 4.60, then the temperature is slowly increased to 120° F. After cooking, the curd is washed, drained, and evaluated by the procedures outlined by the A.D.S.A. cottage cheese score-card committee.

The quality of curd made by the above method was comparable to curd made in larger vats. Thirty powders were tested for whey protein nitrogen, rennet curd tension, and curd-making. Results demonstrated that neither whey protein, nitrogen, nor rennet curd tension was always reliable for predicting the curd-making property of a powder.

Relationship of bacterial types and numbers to spoilage of pasteurized milk held at certain refrigeration temperatures. W. E. GLENN, University of Kentucky, Lexington.

This study was designed to determine the relationship: *(1)* of the numbers and kinds of bacteria in raw milk to the initial flavor score of the pasteurized product; *(2)* of mesophiles, psychrophiles, and coliforms to the shelf-life of milk stored at 35, 41, and 50° F., and *(3)* of the storage temperature to the shelf-life of the milk.

Results indicate that: *(1)* The numbers and kinds of bacteria in raw milk may affect the initial flavor score of processed milk. *(2)* The bacterial count of the pasteurized milk increases very slowly at 35° F., usually requiring from five to ten days before a significant increase occurs. As the refrigeration temperature is increased, these bacteria grow faster. Preliminary results indicate that 500,000 to a few million organisms per ml. of milk are required before the milk acquires an objectionable flavor. *(3)* Properly processed milk will have low bacterial counts and be free of coliforms and practically free of psychrophiles. Such milk when stored at 50° F. may be expected to have a satisfactory flavor after six days of storage, at 41° F., about 16 days, and at 35° F., about 26 or more days.

Study of the phosphatase test: A preliminary report. P. J. MAGNINO, JR. AND J. B. MICKLE, Oklahoma State University, Stillwater.

Milk was pasteurized at four different temperatures (62.5, 65.0, 71.1, and 76.7° C.). It was tested for phosphatase activity immediately after pasteurization and again after 24 hr. of storage at 32.2° C.

A phenol value of 3.6 ± 1.3 p.p.m. was established as the minmum standard of milk pasteurization. The minimum time of pasteurization was established at the four heating temperatures and these times were found to vary. This was considered to be owing, in part, to seasonal variations and in part to experimental error.

Phenol values obtained before and after incubation were erratic, with more erratic values being obtained after incubation. Most variations obtained were within the experimental error and no general trend toward higher to lower values after incubation was shown.

Salaries of dairy manufacturing graduates. H. B. HENDERSON, University of Georgia, Athens.

Employment opportunities for dairy manufacturing graduates and salaries being received were revealed in a nationwide study in 1954–1955. A continuation of this study revealed that graduates continue to increase their earnings as they gain experience and assume more responsible positions. Preliminary figures on 218 graduates revealing starting salaries and salaries for 1957, and who were employed from 1 to 11 yr., indicate that the average annual increase in earnings has averaged $455. This increase has been maintained rather consistently over the entire period.

Starting salaries, likewise, have increased rather consistently since 1946. The average starting salary for 1946 graduates was $2,980; whereas, for 1956 graduates it averaged $4,250, the highest year of the entire period.

Many graduates report marked improvement in working conditions and attitudes of management. However, many still report poor working conditions, long hours, and no industry training program—as a handicap to satisfactory employment in the dairy industry.

Some factors influencing the time required for dairy cows to consume concentrates. D. V. BOSTAIN AND O. T. STALLCUP, Arkansas Agricultural Experiment Station, Fayetteville.

Data have been obtained on the time required for dairy cows to consume concentrates in four stanchion barns and ten milking parlors under winter-feeding conditions when stored roughages were fed. The minutes required to consume 1 lb. of concentrate for cows in stanchion barns were 2.15 ± 0.89, 2.19 ± 0.84, 3.00 ± 0.96, and 2.41 ± 0.98 for 97 Guernsey, 40 Holstein-Friesian, 49 Jersey, and 186 total cows, respectively. The minutes required to consume 1 lb. of concentrate for cows in milking parlors were 1.77 ± 0.62, 1.53 ± 0.56, 1.89 ± 0.59, and 1.77 ± 0.60, for 61 Guernsey, 52 Holstein-Friesian, 110 Jersey, and 223 total cows, respectively. The difference between barn types and between farms within barn types was significant, indicating that management on the farm has considerable influence on the rate of consumption.

Thirty-two cows of the University herd consisting of 11 Holsteins, 11 Jerseys, six Brown Swiss, and four Guernseys were observed to determine the influence of ration on the time required to consume concentrates. When on a ration of alfalfa hay and sorgo silage, the consumption rate was 1.33 min/lb. When the same cows were receiving high-quality oat-vetch pasture the consumption rate was 2.38 min/lb.

A comparison of two systems of feeding roughages to dairy cattle. A. V. DAVIS AND R. S. WOODWARD, North Louisiana Experiment Station, Calhoun.

Beginning August 1, 1956, and continuing through July 31, 1957, an experiment was conducted to determine the value of a good pasture compared with good-quality alfalfa hay. Two equal groups of ten Jersey cows were used. Group 1 was placed on the best pasture available, supplemented with home-grown grass hay; whereas, Group II received a limited amount of very poor pasture and good-quality alfalfa hay. Grain feeding was the same for each group.

Group I produced an average of 24.57 lb. of 4% FCM per cow-day of production, valued at $1.43. Group II produced an average of 23.98 lb. of 4% FCM per cow-day of production, valued at $1.40. Cost of production for Group I was $0.48 per cow per day, and for Group II, $0.55. The value of production was $0.03 per cow per day greater, and the cost of production was $0.07 per cow per day less, for Group I. Thus, the value of a good pasture program over a poor pasture supplemented with good-quality alfalfa hay was $0.10 per cow per day. The cows in Group I gained 53.9 lb. per cow; those in Group II, 46.8 lb. per cow.

Substituting concentrates for corn silage in drouth years. W. A. KING, South Carolina Agricultural Experiment Station, Clemson.

In two experiments, 10.0 lb. molasses, 7.3 lb. citrus pulp, and 8.0 lb. ground snapped corn were each substituted for part of the corn silage in the winter ration of milking cows. Hay and silage were fed in outdoor feed bunks and the substitutes were scattered on top of the silage. In the first experiment, the cows were fed an average of 6.8 lb. alfalfa hay, 40 lb. of corn silage, and an 18% crude protein concentrate mixture. Two groups of 75 herd cows each were used in 28-day reversal-type trials. The expeted milk pro-

duction averaged 99.5, 102.5, and 104.3%, respectively.

A continuous feeding trial under better controlled experimental conditions was conducted with four groups of six cows each for 84 days. The cows in the control group consumed 80.3 lb. of corn silage, compared to 53.4 lb. in the three other groups. The average consumption of other feeds was 6.7 lb. alfalfa hay and 13.2 lb. of an 18% concentrate mixture. The expected milk production averaged 106.1% for the controls and 105.4, 104.7, and 105.0%, respectively, for the other rations. The milk production averaged 42.7, 42.9, 42.9, and 43.0 lb. per cow daily, respectively.

A Johnson grass–Sorghum hybrid, Coastal bermuda grass, and Tift Sudan as pastures for milk production. W. D. Craft, J. R. Owen, W. C. Cowsert, and J. T. Miles, Mississippi Agricultural Experiment Station, State College.

This trial was carried out during the summer of 1957, using a Latin-square design with three groups of six cows each. Three two-acre paddocks of each crop were used. Additional cows were added to the group of 18 tester cows, when needed to keep the crops properly grazed. The pastures were evaluated by measuring milk production and change in body weight of tester cows and obtaining the carrying capacity per acre of each grass.

The average daily milk production per cow was 36.4 lb. on the Johnson grass–sorghum hybrid, 32.1 lb. on the Coastal bermuda grass, and 36.1 lb. on Tift Sudan. Average daily changes in body weights were ± 0.39, ± 0.12, −0.18 lb., respectively, for the three crops.

The total cow-days grazing provided were 124.2 days per acre for the hybrid, 256.3 days for Coastal bermuda, and 123.8 days for Tift Sudan. The carrying-capacity measurements on the hybrid and Coastal bermuda grass covered the entire summer-growing season. The Sudan plots were prepared and seeded between April 13 and 30, following oat grazing, and were replanted in oats in mid-September. The cow-days of grazing on two of the hybrid plots were somewhat affected by crabgrass competition.

Comparison of Georgia Number One hybrid millet and Piper sudan for lactating dairy cows. L. J. Boyd and J. B. McLaren, Tennessee Agricultural Experiment Station, Knoxville.

A reversal-type experiment involving 15 pairs of Holsteins was conducted during the summer of 1957, in which every cow was fed each of the two forages for a 28-day period. The forages were chopped daily and fed in a dry-lot. The paired mates were fed the same amount of grain daily, and hay was allowed to each group at the rate of 3 lb. per cow per day.

During the first period. the average daily milk production for the Millet group was 35.8 lb., and 33.8 lb. for the Sudan group. Group data revealed that an average of 181 lb. of Millet and 163 lb. of Sudan were consumed per cow per day during this period. The average daily production during the second period was 29.3 and 29.2 lb. for the cows on Millet and Sudan, respectively. During this second period, when forage quality was lower, average daily consumption dropped to 98 lb. for Millet and 103 lb. for Sudan.

There was no significant difference between the two groups in the production of milk or butterfat. None of the cows showed abnormal changes in butterfat test. Changes in body weight were the same for both groups.

The feeding value of oat silage cut at two stages of maturity and preserved with sodium metabisulfite or ground snap corn. L. R. Sisk, M. E. McCullough, and O. E. Sell, Georgia Experiment Station, Experiment.

Oats cut in the prebloom and boot stages were ensiled in upright silos during two growing seasons. Both years, two silos were filled with alternate loads of prebloom oats, using $Na_2S_2O_5$ in one and ground snap corn in the other. Preservatives used: Silo 1, $Na_2S_2O_5$; Silo 2, ground snap corn; Silo 3, $Na_2S_2O_5$, and Silo 4, ground snap corn. For each silage, cows were grouped according to level of production and body weight and group-fed for 28-day periods. Average daily intakes of the silages were 75.6, 82.3, 84.5, and 80.6 lb. for Silos 1, 2, 3, and 4, respectively. Between the two preservatives used, a significant difference was observed in milk production. Oats preserved with ground snap corn held milk persistency to a 6% loss in 1956 and a 9% loss in 1957. The $Na_2S_2O_5$ silage showed a loss of 22 and 26% for the two years. The prebloom stage resulted in equally good milk production without the excessive storage losses encountered in the boot-stage oats. Information on silage losses and other factors also will be presented.

Saccharin supplementation in the diet of ruminants. L. L. Rusoff, W. J. A. Payne, E. J. Stone, and G. A. Hindery, Louisiana Agricultural Experiment Station, Baton Rouge.

Sweetening agents, such as molasses and sugar, have been used with success in rations for farm animals. Saccharin is much sweeter than sugar, although regarded as having no food value. Preliminary experiments were conducted with dairy steers to study the effect of adding saccharin at levels of ½ of 1% and 1% to grain, hay, or silage. Slightly greater consumption of the treated feds was obtained.

In another study, various levels of saccharin

(¼th lb. to 5 lb.) per ton of oats or millet, were ensiled with and without molasses or sodium metabisulfite in upright concrete experimental silos. Palatability studies using dairy steers showed that the greatest consumption of silage was obtained with the mixture of ¼th lb. or ½ lb. of saccharin and 35 lb. molasses, per ton. Animals also consumed more silage when it was treated with saccharin than when it was treated with bisulfite.

In a 20-day study conducted during hot weather, the addition of 0.05 lb. of saccharin per 100 lb. of water increased the water consumption and body weights of dairy cows.

Some results of measuring cellulose digestion of forages by small-sample techniques. J. W. LUSK AND J. T. MILES, Mississippi Agricultural Experiment Station, State College.

The digestibility of the cellulose of several forages was determined concurrently by suspending small silk bags in the rumen of fistulated cows and by a slight modification of the Cheng, Hall, and Burroughs artificial-rumen technique. Average digestibility of cellulose in beet pulp was 92.2%, in alfalfa hay, 50.1%, in Johnson grass hay, 62.0%, and in Coastal bermuda hay, 59.5%, by the in vivo method. In a conventional digestion trial, 55% of the cellulose in the same sample of alfalfa hay was digested.

Initially, an international centrifuge at 2,000 r.p.m. (1133 × G), as described by Lambert and Jacobson, was used in obtaining inoculums for the artificial rumen. Results were quite variable. When a high-speed angle centrifuge was used at 5,000 r.p.m. (3193 × G), results were repeatable. In a series of carefully controlled trials, digestion of the cellulose in four feeds averaged 55%, using inoculums obtained at slow speed; and 62% for high speed when the bacterial cultures were not washed, and 16 and 57%, respectively, when the cultures were washed twice. A feed-by-speed interaction was present on both the washed and unwashed cultures.

Proximate analysis of lignin isolated from various roughages. O. T. STALLCUP, Arkansas Agricultural Experiment Station, Fayetteville.

Lignin was isolated from various dry roughages, then subjected to a proximate analysis as outlined by the A.O.A.C. methods. The crude protein, ether extract, crude fiber, ash, and N.F.E. contents, respectively, of the lignin of various roughages (dry matter basis) were as follows: Alfalfa hay—1.24, 3.08, 39.40, 5.20, and 51.08%; Bermuda grass hay—9.55, 2.73, 38.64, 17.87, and 31.21%; oat straw—3.55, 1.04, 51.06, 9.21, and 35.14%; Kentucky 31 Fescue hay—3.57, 2.90, 48.73, 8.49, and 36.31%; orchard grass hay—10.52, 0.94, 23.70, 10.03, and 54.81%; upland prairie hay—7.12, 3.05, 14.08, 14.23, and 61.52%. These data indicate that the character of lignin varies from one plant source to another. Nitrogen, ether extract, and minerals are trapped in varying degrees by lignin, depending on the plant source. These data also indicate that in some plants more of the lignin appears in the nitrogen-free extract fraction than in the crude fiber fraction. These data lend credence to the facts that crude fiber does not comprise all of the indigestible carbohydrates, nor does the nitrogen-free extract represent only digestible carbohydrates.

Utilization of wood pulp as a roughage replacer in experimental dairy rations. G. F. WILLIAMS, S. D. MUSGRAVE, J. D. SCHUH, AND R. W. MACVICAR, Oklahoma State University, Stillwater.

Eleven digestion trials have been completed in which wood pulp cellulose[1] was used as a roughage replacement. Four groups of dairy bulls and one group of dairy steers, totaling 26 animals, were used. They averaged from 225 to 860 lb. For three years, 14 of the bulls have been on measured-intake rations, in which wood pulp was the only roughage offered.

For rations containing wood pulp and a well-balanced grain mixture, apparent TDN coefficients have exceeded 68%. For two trials in which the only constituents were wood pulp, cottonseed meal, and mineral and vitamin supplements, apparent TDN coefficients dropped to 54%. Apparent crude fiber digestion was 40% or more in all trials.

When wood pulp was mixed with molasses, cottonsed meal, or grains, animals readily consumed amounts equal to the accepted recommended amounts of dry matter. Limited acceptance of the dry wod pulp occurred when it was fed alone.

Animals made satisfactory growth while receiving wood pulp supplemented with a well-balanced grain mixture. Lowered rumen activity and a high rumen pressure were observed. Results of one digestion trial in which the animals received only wood pulp, minerals, and vitamins will be presented.

[1] Trade name "Solka Floc", Brown & Company.

Effect of environmental temperatures on milk production. J. E. JOHNSTON, Louisiana Agricultural Experiment Station, Baton Rouge.

Recent research at L.S.U. indicates that the effect of high environmental temperatures on milk production can be divided into two subsidiary effects: *(1)* the direct effect of hot conditions on the animal, and *(2)* the indirect effect mediated through changes in nutritional regimen. The nutritional effect is owing to changes in forage quality associated with prolonged hot weather, and results

in a depression of forage digestibility and milk production. This can be prevented, or the effect limited, by the use of supplemental forages, irrigation and, perhaps, use of stored roughages. The direct effect of hot conditions on the animal depends, to a great extent, upon the prior acclimatization of the animal. When dairy cows are subjected suddenly to hot weather without prior adaptation, the immediate result is a depression of milk production and feed consumption concomitant with a marked rise in body temperatures and respiration rates. If, however, the cows have previously been adapted by a gradual increase in temperature and humidity, these same hot conditions have very little effect upon milk production and feed consumption. Prolonged exposure to hot conditions results in a gradual decline in milk production, apparently associated with adaptive metabolic changes.

CALCIUM ION CONCENTRATION IN MILK

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SUMMARY

The murexide method of Smeets *(15)*, modified to give increased sensitivity and coupled with a pressure ultrafiltration technique that provides sufficient sample in approximately 1 hr., is a simple, convenient method for the determination of calcium ion in milk on a semiroutine basis. With suitable adjustment of the ionic strength and sodium content of the reference solutions, the method is applicable also to concentrated milk products.

Skimmilk was found to contain from 2.5 to 3.4 m*M*/liter Ca^{++}. Addition of calcium to milk precipitated both phosphate and citrate but increased $[Ca^{++}]$; addition of phosphate precipitated calcium and decreased $[Ca^{++}]$, and addition of citrate dissolved colloidal phosphate but decreased $[Ca^{++}]$. The apparent solubility products of di- and tricalcium phosphate in milk appear to be approximately 1.5×10^{-5} and 1×10^{-21}, respectively.

Heating skimmilk caused precipitation of calcium phosphate and lowered $[Ca^{++}]$. Concentration of milk also caused precipitation of calcium phosphate, but $[Ca^{++}]$ increased.

Although the relations between calcium ion concentration ($[Ca^{++}]$) and other properties of milk are not obvious *(3, 6)*, $[Ca^{++}]$ is probably one of the factors controlling the characteristics of milk colloids. $[Ca^{++}]$ must also be related to the solubility of the calcium phosphate salts present in the milk colloids. Several methods for the estimation of calcium ion concentration in milk have been published recently *(1, 3, 6, 14, 15)*. Of these, the murexide method *(15)* appeared to be the most simple and direct, but this method required some modification before it could be applied to concentrated milk products. Therefore, a modified method was developed, together with a rapid ultrafiltration procedure. Details of these methods, and additional data on the relation between $[Ca^{++}]$ and total calcium, phosphate, and citrate are presented.

MATERIALS AND METHODS

Morning milk was separated raw at about 34° C., and the skimmilk pasteurized at 66° C. for 30 min. Ultrafiltrates were prepared from 100-ml. lots of milk at room temperature by filtration at 30 p.s.i.g. through 1-in. diameter dialysis tubing (Cellophane) supported by stainless steel screens (Figure 1). Ultrafiltrate was collected in a large test-tube which totally enclosed the screen so as to reduce evaporation. Approximately 10 ml. of ultrafiltrate were collected per hour.

For the determination of ionic calcium all glassware was rinsed in 1:40 hydrochloric acid. Standard solutions were prepared in glass-distilled water and stored in polyethylene bottles. Murexide (ammonium purpurate, Eastman

Received for publication October 23, 1957.

NRC No. 4660.

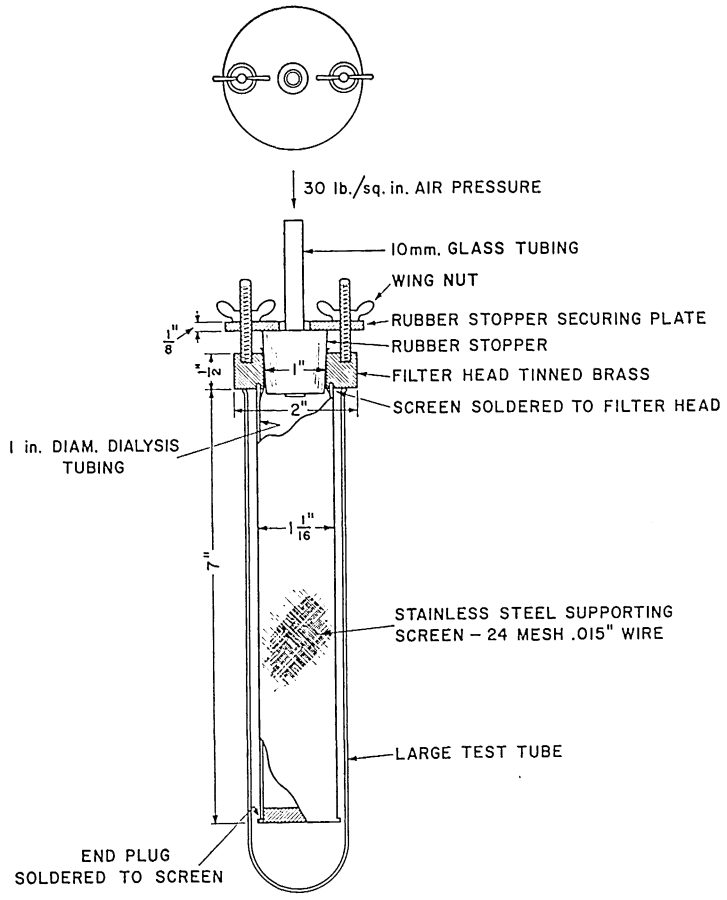


FIG. 1. Apparatus for rapid ultrafiltration of milk.

Kodak Co.), prepared as a 0.1% solution in water, was stable for several days in polyethylene at 4° C. Standard calcium solutions were prepared from reagent grade calcium carbonate and a slight excess of hydrochloric acid, with subsequent neutralization to pH 6.8 with potassium hydroxide.

The procedure finally adopted for the determination of $[Ca^{++}]$ was to place 0.1 ml. of murexide solution in a test-tube, add 4 ml. of milk ultrafiltrate, and determine the absorption at 520 and 480 mμ with a Carry[2] recording spectrophotometer. A second aliquot of ultrafiltrate served as the reference blank. Calcium standards at appropriate potassium : sodium ratios and ionic strength were prepared at the same time, and the calcium content of the ultrafiltrate estimated from a plot of the $E'_{520} - E'_{480}$ (vide infra) values. Readings could be made immediately after addition of the samples or delayed up to 2 hr.

Total calcium was determined by a turbidimetric potassium oleate method *(7)*, phosphate by a colorimetric phosphomolybdate method *(8)*, and citrate by a

[2] An Evelyn colorimeter can be substituted for the recording spectrograph by adding 10 ml. of sample to 0.1 ml. of murexide and reading with No. 515 and 470 filters.

modification of the acetic anhydride-pyridine method *(11)*, in which trichloracetic acid was replaced by 0.1 *N* hydrochloric acid.

The concentration of specific salts in milk was increased by adding up to 1.5% by volume of molar solution of calcium chloride (neutralized to pH 6.7 with potassium hydroxide), or potassium phosphate (pH 6.7) or sodium citrate (pH 6.8) to the milk. The dilution of each sample, including the control, was kept constant by appropriate additions of distilled water.

DEVELOPMENT OF THE CALCIUM ION METHOD

The absorption maximum of a murexide solution changes progressively from 520 to 480 mμ as the [Ca^{++}] is increased *(12, 15)*. Rafflaub *(10)* estimated calcium ion concentration from the extinction coefficient of 470 mμ with reference to standard calcium solutions. Smeets *(15)*, following Schwarzenbach and Gysling *(12)*, determined an "average dissociation constant" and calculated [Ca^{++}] of unknown solutions by means of this factor. To determine the most suitable procedure, the whole absorption curve was recorded for each solution and various methods of relating absorption readings to [Ca^{++}] were tried. A plot of the absorption at a single wavelength against [Ca^{++}] gave curves that were relatively flat at calcium levels above 2 m*M*/liter (Figure 2). A plot of wavelength of the absorption maximum against [Ca^{++}] gave a similar curve, but had the advantage of being independent of murexide concentration. Increased sensitivity was obtained by plotting the value obtained by subtracting the ab-

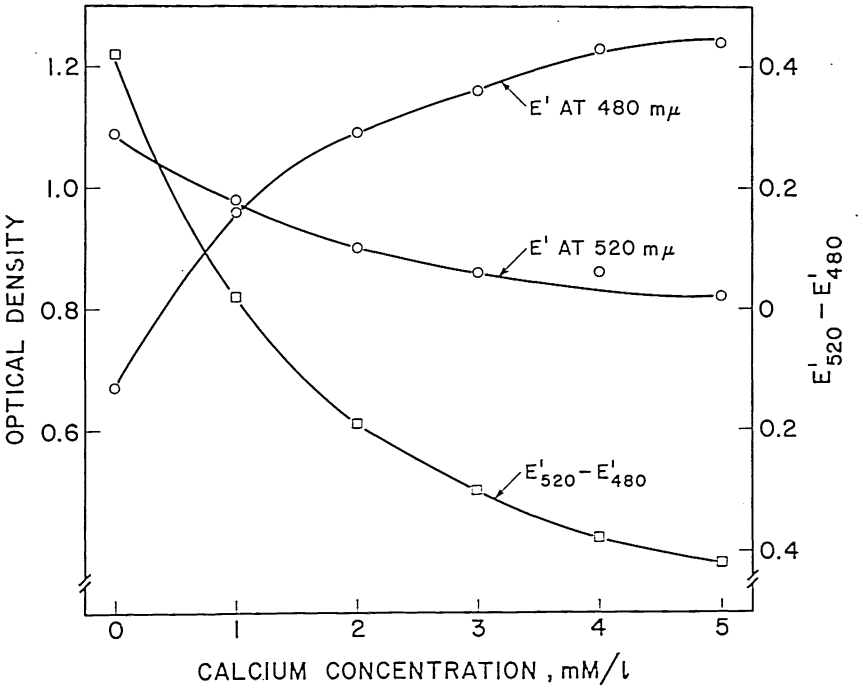


FIG. 2. Light absorption of murexide at various calcium levels (0.1 ml. murexide, 4 ml. calcium solution, $\mu < 0.01$).

sorption at 480 mμ from that at 520 mμ ($E'_{520} - E'_{480}$ value) against [Ca^{++}] (Figure 2), and at murexide concentrations of about 0.1 mgm/4 ml. sample this curve was only slightly affected by murexide concentration. It has, therefore, been used throughout this work.

In agreement with the results of Smeets *(15)*, lactose and magnesium did not interfere with the estimation of [Ca^{++}], citrate reduced [Ca^{++}] in accordance with the known dissociation constant *(4)*, and phosphate reduced it slightly. Therefore, no further study of these factors was undertaken.

On the other hand, ionic strength and sodium ions markedly affected the results. Increasing ionic strength with potassium chloride had only a moderate effect on the absorption of murexide in the absence of calcium but, in the presence of calcium, increasing the potassium shifted the absorption curve of calcium murexide towards that of murexide alone (Figure 3). This effect persisted throughout the range of measurable calcium concentrations.

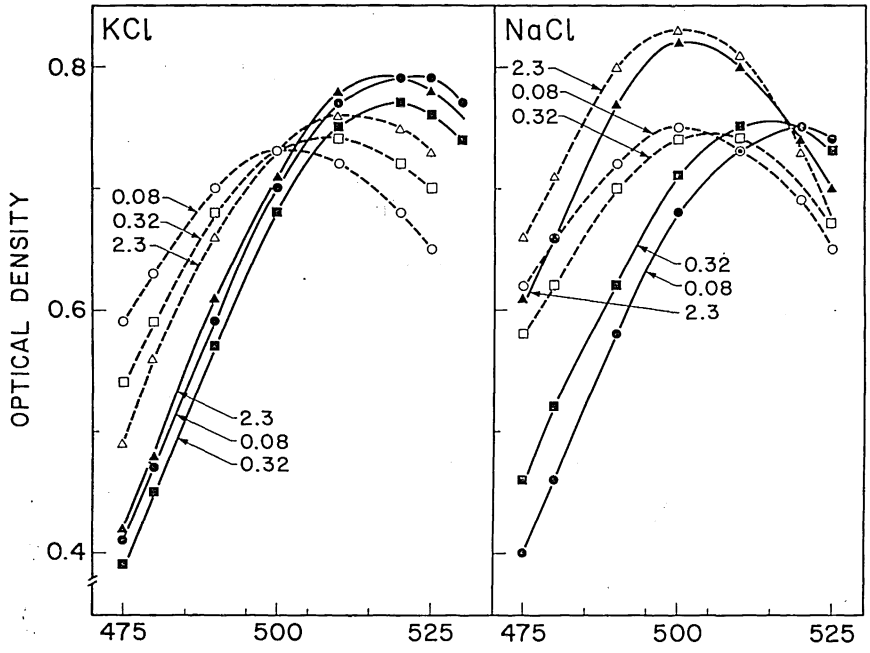


FIG. 3. Effect of potassium and sodium chloride on the light absorption of murexide. Solid lines—no calcium; dotted lines—2 m*M* calcium.

In contrast to potassium, sodium affected the absorption of murexide itself, increasing the absorption maximum and shifting it towards lower wavelengths (Figure 3). In the presence of calcium, sodium had a complex effect, the absorption at 480 mμ, for example, being decreased by increasing sodium up to 0.32 *M*, but being increased again by 1.0 or 2.3 *M* sodium.

Replacing potassium chloride with sodium chloride at fixed ionic strength (Table 1) had only a small effect at μ=0.08, but the differences became progressively greater at higher ionic strengths. Standard curves prepared in the

TABLE 1
Effect of potassium-to-sodium ratio on the light absorption of murexide
(2 m*M* calcium in all samples)

K:Na Ratio	$E'_{520}-E'_{480}$ at ionic strength			
	0.08	*0.32*	*1.0*	*2.3*
All K	+0.05	+0.13	+0.18	+0.23
3K:1Na	+0.04	+0.12	+0.15	+0.15
1K:1Na	+0.03	+0.10	+0.12	+0.10
1K:3Na	+0.03	+0.08	+0.09	+0.05
All Na	+0.03	+0.08	+0.06	−0.01

presence of potassium alone are thus acceptable for work with normal milks, but the potassium-to-sodium ratio found in the samples must be approximated in the standard solutions when [Ca^{++}] is determined in concentrated milk products.

RESULTS AND DISCUSSION

Application of the original method *(15)* and our modified method to ultrafiltrates from normal milks gave values that did not differ by more than 0.1 m*M* Ca^{++}/liter. Reproducibility of results obtained with the modified method appeared to be approximately ±0.05 m*M* in normal milks and ±0.1 m*M* at higher calcium levels. Determination of [Ca^{++}] in a synthetic mixture resembling protein-free milk serum gave a value of 2.2 m*M*, whereas calculation from published dissociation constants for calcium citrate *(4)* gave a value of 2.31 m*M*.

Values for a series of skimmilks tested over a 2-yr. period ranged from 2.5 to 3.4 m*M* Ca^{++}/liter. This range is slightly higher than those given by van Kreveld and van Minnen *(6)* and by Christianson *et al. (3)*, but agrees with that of Smeets *(15)* and is lower than the values reported by Affsprung and Gehrke *(1)*.

The gradually increasing concentration of milk colloids within the membrane during ultrafiltration did not detectably affect [Ca^{++}] in three consecutive 10-ml. samples of ultrafiltrate taken from one 100-ml. aliquot of milk. Whole and skimmilk yielded ultrafiltrates with the same [Ca^{++}], thus confirming the results of van Kreveld and van Minnen *(6)*.

Addition of calcium to skimmilk before ultrafiltration caused an increase in [Ca^{++}] and total calcium, and a decrease in total phosphate and citrate in the ultrafiltrate (Table 2). Smeets *(15)* observed a similar decrease in total phos-

TABLE 2
Effect of the addition of calcium to skimmilk on composition of the ultrafiltrate
(All samples adjusted to pH 6.57 after addition of calcium)

Calcium added	[Ca^{++}]	Total Ca	Total PO_4	Citrate	$[Ca^{++}][HPO_4^{=}]$	$[Ca^{++}]^3[PO_4^{\equiv}]^2$
	(mM/liter)					
0	3.4	9.9	9.9	10.5	1.3×10^{-5}	4.3×10^{-23}
2.5	4.1	10.3	8.9	10.2	1.4×10^{-5}	6.1×10^{-23}
5.0	5.3	11.7	7.8	10.1	1.6×10^{-5}	10.1×10^{-23}
7.5	6.5	11.6	7.0	9.6	1.8×10^{-5}	15.1×10^{-23}
10.0	7.2	11.5	5.7	9.2	1.6×10^{-5}	13.5×10^{-23}

phate and, on the assumption that only dicalcium phosphate precipitated, calculated the "change in protein-bound calcium." The data (Table 2) show that citrate also decreased, but because tricalcium phosphate also may have precipitated, calculation of protein-bound calcium is not possible.

Addition of phosphate to skimmilk decreased [Ca^{++}] and total calcium in the ultrafiltrate (Table 3), and addition of citrate to skimmilk decreased [Ca^{++}] and increased total calcium, total phosphate, and pH of the ultrafiltrate (Table 4).

TABLE 3

Effect of the addition of phosphate to skimmilk on composition of the ultrafiltrate

(All samples adjusted to pH 6.62 after addition of phosphate)

Phosphate added	[Ca^{++}]	Total Ca	Total PO_4	[Ca^{++}][$HPO_4^{=}$]	[Ca^{++}][$PO_4^{\equiv}$]^2
	(mM/liter)				
0	2.8	9.2	9.6	1.1×10^{-5}	3.3×10^{-23}
5	2.5	8.9	14.1	1.5×10^{-5}	5.1×10^{-23}
10	2.3	8.2	19.5	1.9×10^{-5}	7.4×13^{-23}
15	2.1	7.4	23.2	2.0×10^{-5}	8.0×10^{-23}

TABLE 4

Effect of the addition of citrate to skimmilk on composition of the ultrafiltrate

Citrate added	pH of ultrafiltrate	[Ca^{++}]	Total Ca	Total PO_4	[Ca^{++}][$HPO_4^{=}$]	[Ca^{++}]^3[$PO_4^{\equiv}$]^2
(mM/liter)			*(mM/liter)*			
0	6.72	2.4	7.8	9.0	1.0×10^{-5}	3.7×10^{-23}
5	6.82	1.9	9.8	10.2	1.0×10^{-5}	4.7×10^{-23}
10	6.90	1.8	10.9	11.1	1.1×10^{-5}	8.1×10^{-23}
15	7.02	1.8	13.1	12.8	1.5×10^{-5}	23.2×10^{-23}

The calculated values for [Ca^{++}] [$HPO_4^{=}$] (k_2) and [Ca^{++}]^3 [$PO_4^{\equiv}$]^2 (k_3), assuming all of the phosphate in ionic form,[3] are given (Tables 2, 3, and 4). The average value of k_2 in the initial samples (skimmilk plus 1.0 or 1.5 ml. of water per 100 ml.) is 1.1×10^{-5}, and the range for all samples, regardless of calcium, phosphate, or citrate addition is from 1.0 to 2.0×10^{-5}. The average value for k_3 in the initial samples is 3.8×10^{-23} (note the higher initial value in Table 6), and the range for all samples is from 3.3 to 23.2×10^{-23}. Precipitation of calcium salts occurred in samples with added calcium or phosphate, whereas solution of colloidal phosphate occurred in the samples with added citrate; therefore, it can be assumed that most of these samples were saturated with calcium phosphate. The variability of both k_2 and k_3 corresponds approximately to a two-fold variation in [Ca^{++}] at fixed phosphate concentration and pH; therefore,

[3] Dissociation constants of $K_1^1 = 1.154 \times 10^{-2}$, $K_2^1 = 1.68 \times 10^{-7}$, and $K_3^1 = 2.34 \times 10^{-12}$ were calculated from the equation of Hentola (*Chem. Abstr.*, 44: 5192i. 1950.) for a potassium chloride solution of ionic strength 0.08. These data were selected because they give all three constants under a single set of conditions approximating those of milk. The K_2^1 value is also in good agreement with that obtained in the more extensive work of Bates and Acree (*J. Research Natl. Bur. Standards*, 34: 373-394. 1945).

no conclusion can be drawn regarding the nature of the precipitated salt. Errors involved in estimating the composition of precipitated salt by difference are too great for the calculation to be significant. However, the data do suggest that the solubility products for di- and tricalcium phosphate in milk are roughly 1.5×10^{-5} and 1×10^{-24}, respectively. These values are much higher than those reported under other conditions *(5)*, but Boulet and Rose *(2)* have pointed out that published values can not be applied to milk.

Heating skimmilk to 66° C. for 30 min. lowered total calcium and phosphate in the ultrafiltrate obtained approximately 1 hr. after recooling to room temperature, but [Ca^{++}] did not change significantly (Table 5). Heating to 82° C. for

TABLE 5

Effect of heating milk and ultrafiltrate on composition of the ultrafiltrate

Treatment	pH	[Ca^{++}]	Total Ca	Total PO_4	Total citrate
Milk heated before ultrafiltration			*(mM/liter)*		
Control	6.79	2.6	8.6	9.9	7.8
66° C., 30 min.	6.72	2.7	7.5	9.2	7.7
82° C., 30 min.	6.70	2.4	6.8	8.5	7.7
Ultrafiltrate heated [a]					
Control	6.63	2.9	7.1	10.3	7.6
66° C., 30 min.[b]	6.59	2.5	7.0	10.3	7.4
82° C., 30 min.[b]	6.36	1.6	5.2	8.9	7.4

[a] The ultrafiltrate was not prepared from the same milk as used in the "Milk" part of the table.

[b] Centrifuged at 28,000 r.p.m., No. 30 head, Spinco preparatory centrifuge, to remove finely dispersed precipitate.

30 min. lowered total calcium and phosphate still further and lowered [Ca^{++}] by 0.2 m*M*/liter. Van Kreveld and van Minnen *(6)* reported similar changes after heating to 95° C. for 3 min., and Christianson *et al. (3)* report a 0.6 m*M* loss after heating to 85° C. for 30 min.

Heating the ultrafiltrate itself caused the formation of a finely dispersed precipitate and a decrease in [Ca^{++}] (Table 5). Removal of the finely dispersed precipitate by high-speed centrifugation (66,000 × G for 1 hr.) decreased total calcium and phosphate without further changes in [Ca^{++}].

Concentration of skimmilk by low-temperature evaporation (21° C.) increased [Ca^{++}] proportionately less than it increased total calcium or phosphate (e.g., [Ca^{++}] in 3:1 milk—4.4 m*M*/liter, total calcium 23.2 m*M*/liter, and total phosphate 28.8 m*M*/liter. Concentration of the ultrafiltrate itself, followed by centrifugation to remove precipitated salts and lactose, decreased pH and increased the concentration of all salts (Table 6). Citrate concentration increased essentially to the same extent as sodium or potassium, indicating that no calcium citrate precipitated. Calcium phosphate, on the other hand, precipitated quite extensively, although the solubility of these salts as indicated by [Ca^{++}] [$HPO_4^{\equiv}$] and [Ca^{++}]^3 [$PO_4^{\equiv}$]^2 was increased more than tenfold by the increasing ionic strength.

TABLE 6

Effect of concentrating an ultrafiltrate

Approximate concentration	pH	$[Ca^{++}]$	Total Ca	Total PO_4	Citrate	Potassium	Sodium	$[Ca^{++}][HPO_4]$	$[Ca^{++}]^3[PO_4\equiv]^2$
		(mM/liter)							
Normal	6.71	2.8	7.8	11.2	8.9	37.8	16.5	1.5×10^{-5}	8.5×10^{-23}
3.6:1	6.26	6.4	28.0	37.0	32.0	134.0	61.0	5.5×10^{-5}	36.0×10^{-23}
11:1	5.84	15.4	59.0	100.0	94.0	405.0	183.0	15.6×10^{-5}	104.0×10^{-23}

ACKNOWLEDGMENTS

Thanks are extended to the staff of the Dairy Technology Laboratory, Central Experimental Farm, Ottawa, for their cooperation in providing milk for these tests, and to Mr. R. Cyr for technical assistance.

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HARDNESS OF BUTTER. I. INFLUENCE OF SEASON AND MANUFACTURING METHOD[1]

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SUMMARY

Hardness was measured on weekly samples of conventionally churned and continuously made butter,[2] taken over a period of 1 yr. Penetrometer and sectility methods were used at 17 and 12° C., respectively. The continuously made butters were consistently harder than the conventionally churned butters. Similar seasonal variations were found for both butters and were greater than differences between the two butter types at 17° C. Hardness maxima occurred simultaneously with softening-point minima, as determined previously. Hardness of conventional butter was less influenced by the experimental temperature difference than was that of the continuous type butter. The hardness of the continuous type butter decreased as gas was added and, therefore, gas content may be a partial cause of the hardness differences between the two butter types. A major portion of the setting of the continuous butter occurred during the first 3 hr. after manufacture and was attributed to crystallization, which was still in progress. The hardening taking place after this first period was attributed to thixotropic changes.

Extensive hardness increase, assumed to be caused by thixotropic changes, occurred in conventional butter and extended over approximately 1 wk. Setting was prevented in both butters during storage at low temperature (−20° C.), but resumed a normal course on subsequent removal of the butter to higher storage temperatures. A permanent hardness decrease was imparted to both butter types by storage at 20-22.5° C.

It has been shown that butterfat in this region is subject to considerable seasonal variation in chemical composition *(13)*, and it is a well-known fact that such changes will profoundly influence the hardness of butter. In a preliminary investigation, continuously made butter was found to be harder than conventional butter *(14)*. The difference was attributed to the remarkable dissimilarity in crystalline structure, but differences in gas content of these two types of butter also may be partly responsible.

Conventional butter increases considerably in hardness subsequent to removal from the churn. This phenomenon is generally called setting, and has been attributed to thixotropic changes. When a study is made of the influence of manufacturing method on hardness, the setting should be considered an important factor. Storage temperature of the finished butter is also important as a factor influencing the course of hardness changes and should, therefore, receive attention.

The present report is a study of the influence of season and manufacturing method on the hardness of butter. The manufacturing methods chosen were the conventional churning and the fundamentally different continuous method.

Received for publication September 27, 1957.

[1] Financial assistance for this research was provided by the Canada Department of Agriculture, Ottawa, Canada.

[2] Made by a process known as Gold'n Flow, which is copyrighted by the Cherry-Burrell Corp., Chicago, Illinois.

METHODS

Hardness was measured either by the penetrometer method at 17° C. or by the sectility method at 12° C. The penetrometer method was a modification of the procedure described by Kruisheer and den Herder *(7)*. The disk plunger of 4 cm.^{2} surface area was mounted in an apparatus similar to the Swift consistometer described by Clardy *et al. (1)* and moved at 5 cm/min. Each butter sample was prepared for hardness measurement immediately after manufacture, by being pressed into a square frame of 5-cm. side and 2.9-cm. height, fabricated from a 20-gauge stainless steel sheet. For certain experiments, it was necessary to use continuous type butter from the experimental continuous butter-making machine developed in this Department *(15)*, and for this purpose the butter was extruded directly into 2.9-cm. lengths of 2-in. O.D. stainless steel tubing.

At lower temperatures, the resistance to penetration exceeded the range that could be recorded by the gauge of the apparatus, and a sectility method was substituted. This was achieved by replacement of the disk by a taut stainless steel wire (diameter 0.015 in.) held in a frame. The gauge reading was taken when the wire had travelled 2.5 cm. through a block of butter which had first been pushed from its frame.

The butter samples, unless otherwise stated, were kept in the frames at 5-10° C. for 1 wk., then tempered in a water bath for 24 hr. prior to testing. Water bath temperatures of 17° C. in the penetrometer method and 12° C. in the sectility method were maintained thermostatically within ±0.05° C., and measurements immediately followed removal of the samples from the water bath, in a room having a temperature in the range 5-10° C.

Each hardness value reported, unless otherwise specified, is the average of two determinations. Penetrometer hardness is expressed as kg/4 cm.^{2}, whereas sectility is expressed as kg/5 cm., since 5 cm. was the length of the wire in contact with the butter.

RESULTS

Accuracy of methods. To test the accuracy of the methods, ten replicate samples of continuously made and ten of conventional butter were measured by both penetrometer and sectility methods. From the results (Table 1), it appears that the accuracy of the methods is very satisfactory.

TABLE 1

The accuracy of hardness measurements

	Conventional butter		Continuous butter	
	Penetrometer method	Sectility method	Penetrometer method	Sectility method
Range [a]	2.00 — 2.12	0.24 — 0.26	3.94 — 4.14	1.02 — 1.08
Mean	2.05	0.25	4.05	1.05
Av. deviation from mean	± 1.5%	± 4.0%	± 1.4%	± 2.1%

[a] Ten replicate samples of each type of butter.

Round frames gave very slightly higher penetrometer hardness than square frames when the butter was hard, but not when the butter was soft (Table 2).

TABLE 2

Influence of shape of frames on butter hardness values

Description of sample	Penetrometer hardness (*Kg/4 cm.*^{2})	
	Round frames	Square frames
Hard butter [a]	2.40	2.20
Soft butter [a]	0.84	0.84

[a] Average of four samples each.

Seasonal variation. For a period of 1 yr., butter samples were collected weekly from two local creameries. One manufactured butter by the continuous method and the other by the conventional method. The monthly averages of hardness of each butter type at two temperatures are given (Figure 1). It is to be noted that the butter types follow the same general hardness trend throughout the year (for the penetrometer hardness data, r = .81, significant at the 1% level). This indicates that the difference in physical structure is responsible for

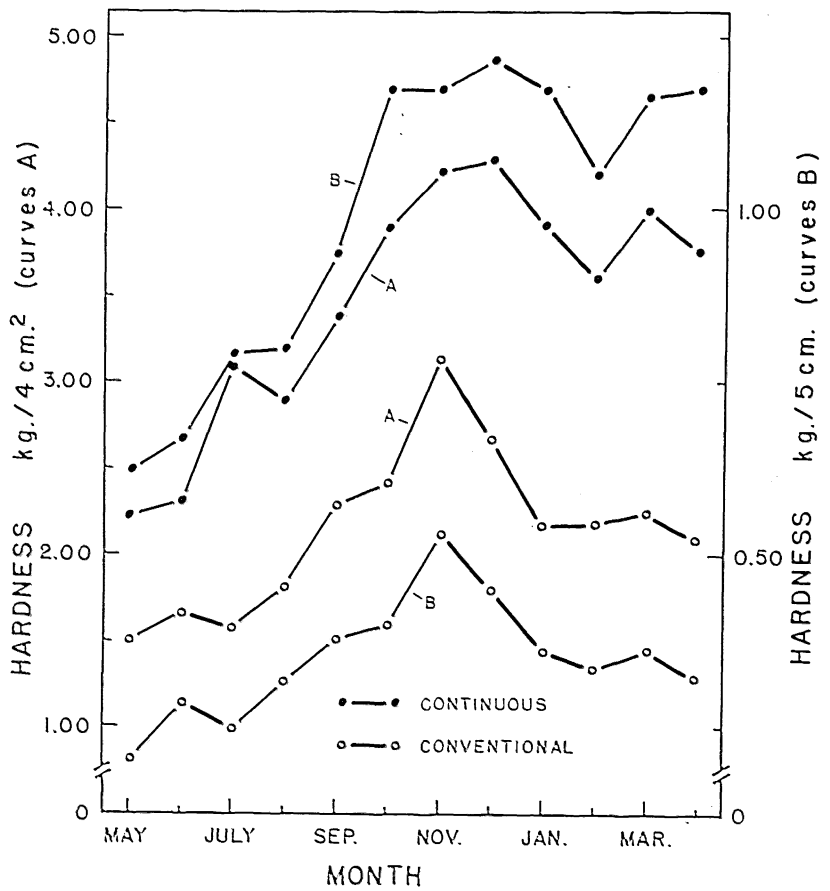


FIG. 1. Comparative hardness of continuous and conventional butter. Curves A: penetrometer method. Curves B: sectility method.

the increased hardness found in the continuous butter, as previous work *(13)* established that the chemical composition of the butterfat of these two Edmonton creameries did not differ significantly. The maximum hardness of both butters occurred in November-December, when the softening point of the butterfat of this region is at maximum value *(13)*; and, conversely, minimum hardness of the butter and minimum softening point of the butterfat coincided seasonally.

The penetrometer and sectility values show a high correlation (for conventional butter, $r = .98$, and for continuous butter, $r = .95$, both significant at the 1% level).

Comparative hardness. It is evident (Figure 1) that on any sampling date the continuously made butter was harder than the conventional butter. The difference was found to be significant at the 1% level.

A remarkable further difference in the hardness properties is revealed in Figure 1. When a point of equal hardness is chosen on the penetrometer curves of each type of butter, it is found that the corresponding sectility values are widely different. The sectility hardness value for conventional butter is then seen to be considerably lower. Thus, the hardness of conventional butter appears to be less influenced by these temperature differences than is the hardness of the continuous butter, since sectility was measured at a lower temperature than was penetrometer hardness.

Influence of gas content. In a preliminary study of the influence of the gas content of butter, samples of continuous type butter with varying nitrogen content were prepared with the experimental continuous machine. The presence of the gas materially lowered hardness values (Table 3).

TABLE 3

Influence of gas content on hardness of experimental continuous type butter

Sample	Gas content	Penetrometer hardness
(No.)	*(%)*	*(Kg/4 cm.²)*
1	0	2.60
	2.0	2.40
	3.6	2.02
2	0	2.48
	1.0	2.28
	16.0	1.68
3	0	2.76
	1.2	2.76
	4.0	1.88
	17.5	1.88

The incidental observation was made, as reported elsewhere *(2)*, that stickiness became apparent in the continuous butter as gas content was increased. The differences in gas content of continuous and conventional butter may explain the differing stickiness of the two butter types.

Setting. A sample of each butter type, taken immediately after completion of manufacture, was maintained in a constant temperature water bath for eight days at the initial temperature, i.e., 5° C. for continuous butter and 12.5° C.

for conventional butter. In this way, the influence of temperature changes on hardness was eliminated. Hardness was measured at these temperatures hourly for 6 hr. and daily thereafter.

Sectility hardness of butter made by the continuous method increased rapidly for the first 6 hr. and less rapidly thereafter (Figure 2). To measure temperature

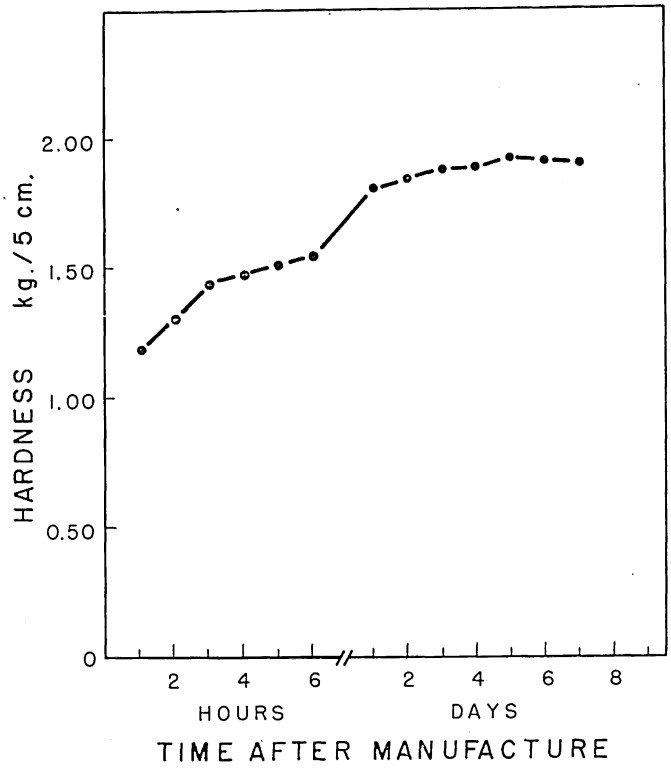


FIG. 2. Sectility hardness values during setting of continuous butter.

changes that accompanied this setting, samples of butter emerging from the texturator were immediately placed in tempered Dewar flasks. The temperature increased from 8.7 to 11.8° C. in 2 hr. (Table 4) and remained fairly constant thereafter.

TABLE 4

Temperatures of continuous and conventional butters stored in Dewar flasks

Type	Temperature ° C.						
	Initial	¼ hr.	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.
Continuous	8.7	10.5	11.1	11.5	11.8	11.9	11.9
Conventional	14.9	15.0	15.0	15.0	15.0	—	—

When conventional butter was held at the temperature that prevailed when the working was completed, 12.5° C., hardness increased for eight days (Figure 3), but the temperature of the butter stored in Dewar flasks remained con-

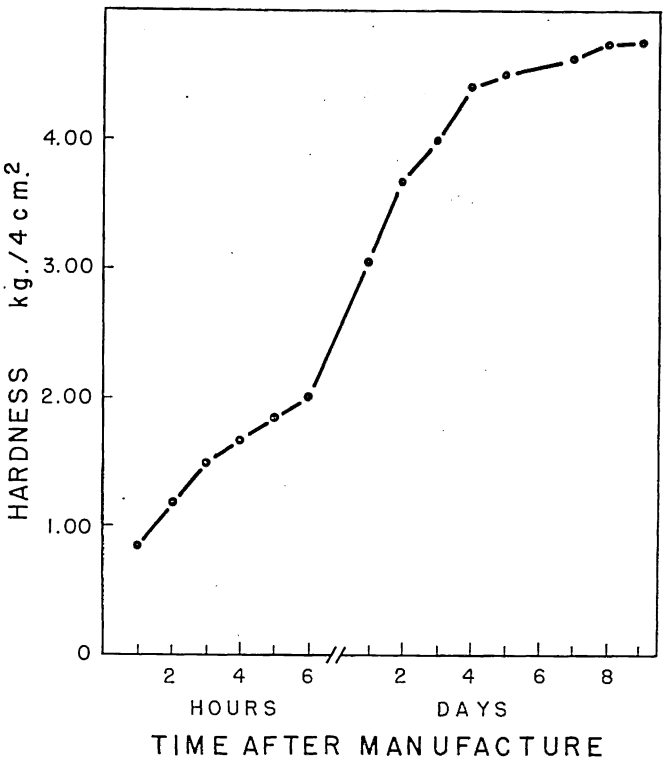


FIG. 3. Penetrometer hardness values during setting of conventional butter.

stant (Table 4). The increasing hardness of conventional butter apparently is a consequence of crystal orientation only, as the crystallization of the butterfat is essentially complete when the working of the butter is finished. In the continuously made butter, however, the hardness increase is largely caused by crystallization, and thixotropic changes are less pronounced.

When replicate samples of each type of butter were stored at 5° C., the penetrometer hardness increase was greater in the conventional butter (Table 5).

TABLE 5

Hardness changes during setting at 5° C.

Days after manufacture	Penetrometer hardness—*(kg/4 cm.²)*	
	Continuous	Conventional
1	3.90	1.76
3	3.96	2.00
7	4.12	2.34

Storage of the butters at −20° C. completely prevented setting, and no measurable hardening occurred. On subsequent removal to 5° C. storage, hardness increased to the same level as in the butters stored at 5° C. immediately following manufacture.

In a further study of the effect of storage temperature, replicate samples

of each butter type were accorded the storage treatments as set forth in Table 6, and penetrometer hardness was measured weekly. To minimize changes related to setting, all samples were stored for the first week at 5° C. Freezing temperature again interrupted hardness increase in each type of butter, but setting was resumed after the butters were removed to 5° C. storage. One week of storage of either butter at 22.5° C. had a remarkable and permanent softening effect. In investigating this phenomenon further, an equivalent softening effect required 2 wk. at 20° C., in place of 1 wk. at 22.5° C., whereas 17.5° C. was ineffective.

TABLE 6

Influence of storage temperature on butter hardness

Butter type	Treatment [a]	Penetrometer hardness—*(Kg/4 cm.²)*, after				
		1 wk.	2 wk.	3 wk.	4 wk.	5 wk.
Continuous	A	2.76	2.84	2.84	2.84	2.88
	B	2.76	2.74	2.74	2.85	2.89
	C	2.76	1.20	1.25	1.18	1.20
Conventional	A	1.88	2.10	2.12	2.16	2.16
	B	1.88	1.86	1.88	2.04	2.14
	C	1.88	1.16	1.18	1.14	1.16

[a] Treatment: A—5 wk. at 5° C; B—1 wk. at 5° C., 2 wk. at −20° C., and 2 wk. at 5° C.; C—1 wk. at 5° C., 2 wk. at 22.5° C., and 2 wk. at 5° C.

DISCUSSION

A large number of methods and instruments has been used for measuring rheological properties of butter. Unfortunately, there is uniformity neither in terminology nor in interpretation of results. Some methods have been adapted to the determination of exact physical properties, e.g., viscosity *(3, 8, 11)*; others have been linked to subjective assessments *(7, 10)*. Hardness of butter is usually measured within the range of 12-17° C., and in choosing measurement temperatures the important consideration is that the range of hardness values encountered falls within the range of the particular instrument used. In this investigation, all samples could be measured by the penetrometer method at 17° C. and by the sectility method at 12° C.

The meaning of the term spreadability is rather vague, and some investigators are of the opinion that spreadability and hardness are not directly related *(10, 12)*; on the other hand, the most widely used methods are of the penetrometer and sectility types.

Hardness has been defined as "a measure of the resistance to permanent deformation or damage" *(9)*. If this definition is accepted, penetrometer, sectility, spreadability, and other methods are all fundamentally hardness measurements, although the results are not always directly comparable.

The high correlation between penetrometer and sectility methods indicates that the same hardness property was measured; whereas, in recently reported work *(5)* on the spreadability and hardness of butter, a highly significant correlation was obtained between spreadability and sectility measurements.

The hardness of butter made by the continuous method, by comparison of corresponding weekly samples, was always significantly higher than that of conventional butter, and similar seasonal changes occurred in the two types of butter. There exists considerable difference in the physical structure of the two butters (crystal structure, gas content, water dispersion), which accounts for the dissimilar hardness. It is to be noted that the penetrometer hardness difference between the two butters does not reach the magnitude of inherent seasonal variations. Dolby *(4)* estimated that up to 80% of differences in hardness of conventional butter were caused by seasonal variations in the composition of the butterfat. Considerable interest has been shown in attempts to influence rheological properties of conventional butter by variation of temperatures before and during churning. The continuous process undoubtedly represents an extreme treatment, as the fat is subjected to rapid cooling and agitation in the chilling compartment, but even this results in penetrometer hardness changes that are low in comparison to seasonal differences.

On the basis of the limited data herein presented, it would be premature to assign to gas content a major role in butter hardness, or to proportion continuous and conventional butter hardness differences between gas content and crystal state.

The extreme hardness increase in conventional butter during the first week after manufacture is attributed to orientation of the crystal particles into a rigid structure, as no indication of crystallization was found during this period. Crystallization is still proceeding in continuous butter as it emerges from the texturator and part of the hardness increase is, therefore, caused by increase in number and/or size of crystal particles.

There was no hardness increase caused by orientation of crystal particles during storage of the butter at low temperature (−20° C.). Contrary to results reported recently *(6)*, the interruption of setting by freezing was found to be not permanent. It is probable that in the aforementioned study the butter was not allowed enough time after freezing to obtain maximum hardness, as suggested by the data reported *(6)*. The influence of setting on hardness deserves further study. At high temperature (20-22.5° C.), the crystalline structure undergoes a breaking-down process and hardness is permanently lowered. It is of interest to note that, at the latter storage temperature, both types of buttter (made at the same time of year) had approximately the same hardness at the end of the experiment.

The role of the state of crystallization in hardness differences will be the subject of further communication.

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OILING-OFF PROPERTIES OF BUTTER[1]

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SUMMARY

In this study, the oiling-off properties of conventionally churned and continuously made butter[2] have been determined.

Butter made by the continuous process had a consistently greater tendency to oil-off at 25 or 28° C. than did conventional butter. Seasonal variations throughout a 12-mo. period followed the same trend for both butter types, and were considerably greater within the type than the differences between corresponding samples of the two types. Maximum and minimum oiling-off occurred in June and October-November, respectively. This corresponds with the dates of minimum and maximum hardness of the butters of this region.

The gas content did not influence oiling-off of the continuous butter, but gas content may contribute to stickiness. Oiling-off, when expressed quantitatively as weight-percentage of the original test sample, was found to be a function of the height of the test block of butter. Temperature, as well as grade and size of filter paper, also influenced the results.

The narrowness of the temperature range in which butter exhibits acceptable physical properties is of paramount concern to the butter industry. Among these properties is that of oiliness resulting from the seepage of free oil from the butter. This condition of the butterfat may manifest itself in a weak, greasy condition of conventionally churned butter, an oily appearance of butter made by continuous methods, and an oily condition of vacuum-churned and worked conventional butter, which is also reported to show droplets of free oil frequently *(11, 12)*. This important characteristic of the texture of butter has not received the attention it deserves, particularly with regard to seasonal variability, influence of butter type, storage temperature, and standardization of measuring procedure. The increasing use of continuous methods and vacuum-churning in butter-making accentuates the need for more extensive information on the physical properties of butter.

In 1924, Hackett and Crowley *(2)* placed butter on filter paper and analysed the oil absorbed at temperatures up to 20° C. Absorption was very slow at the lower temperatures. The oil absorbed at 20° C. contained a higher percentage of unsaturated and short-chain saturated fatty acids than did the oil absorbed at lower temperatures.

Mohr and Bauer *(7)* considered that Storgård's centrifugal method presents experimental difficulty and is unsatisfactory as a measure of oiling-off. They developed the filter-paper procedure into a quantitative method. Oiling-off of summer Alfa butter was reported as being 12.5-17.7%, and of summer conventional butter as being 9.5-11.6%, when measured at 25° C.

Received for publication September 27, 1957.

[1] Financial assistance for this research was provided by the Canada Department of Agriculture, Ottawa, Canada.

[2] Made by a process known as Gold'n Flow, which is copyrighted by the Cherry-Burrell Corp., Chicago, Illinois.

The Mohr and Baur method has been fairly widely used. King by this method found the oiling-off of Swedish Alfa butter (no season reported) to be 11.8-17.5% *(5)*. The Danish range of 5.9-15.7% at 20° C. for conventional summer butter, tempered for packaging directly from the churn *(10)*, is broader than that of Mohr and Baur. The high values at this temperature are not surprising, in view of the fact that the butter is discharged from the churn at an elevated temperature. The major modification of the Mohr and Baur method used by Dolby *(1)* on New Zealand butter at 18° C. prevents comparative evaluation of his results.

METHOD

It is difficult to cut a perfect cube of butter with a stretched wire. For this reason, a special forming device or trier was fabricated from a stainless steel sheet. This trier was about 7 cm. long and had a 2.5 by 2.5 cm. internal cross section. When pushed into the butter at 5° C. and withdrawn, it removed a plug of butter of similar cross section. The butter plug was pushed out of the trier by a closely fitting plunger with an attached graduated stem and the plug was cut with a stretched wire at the 2.5-cm. length, unless otherwise stated.

The cube of butter was placed on a pile of ten Whatman No. 1 filter paper circles of 12.5-cm. diameter and of known weight. The papers and butter cube were weighed and stored at 25 or 28° C. for 48 hr. and the cube then removed. To facilitate removal of the butter, paper and cubes were kept at 8° C. for 10-20 min. During the storage period, the filter papers may absorb some moisture from the atmosphere of the storage. To serve as a control, a pile of ten similar filter papers without a butter cube was stored under the same conditions. After removal from storage, these and the papers with the absorbed oil were held in a dry atmosphere until the control returned to its original weight. The papers with the absorbed oil were then weighed. Oiling-off is expressed as the weight increase of the filter papers in terms of percentage of the weight of the original butter cube.

RESULTS

Continuous vs. conventional butter. For a period of 1 yr., butter samples were collected weekly from two local creameries; one manufactured butter by the continuous method and the other manufactured conventional butter. The weekly samples were tested for oiling-off tendencies and the 25° C. results are presented as monthly averages (Figure 1). There is a marked similarity in the curves for the two types of butter. The differences are within the range 2.0-3.1%, and they average 2.6%. Statistical analysis of the results from duplicate tests of 104 butter samples shows that the difference between the two types of butter is highly significant. It may be assumed that the composition of the fat in corresponding samples of the two butter types did not differ significantly *(13)*. Therefore, differing oiling-off properties were related to unlike physical structures.

Seasonal variability. Maximum and minimum oiling-off occurred in June and October-November, respectively, and the difference between maximum and minimum values is approximately 7% for each butter type (Figure 1). Vari-

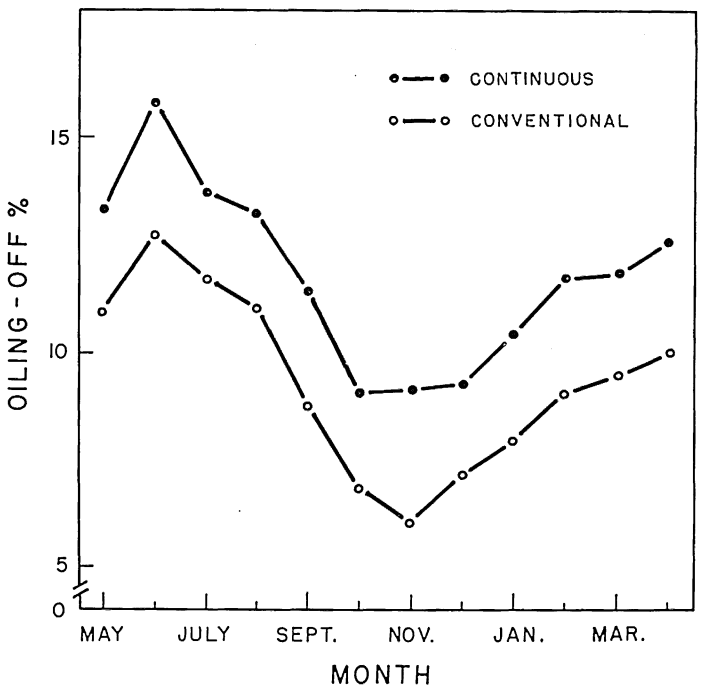


FIG. 1. Comparative oiling-off at 25° C. as influenced by season.

ability of the continuous values was within the range 9.1-15.9%, and of the conventional butters, 6.1-12.8%. Seasonal variations are considerable and of greater magnitude than differences caused by dissimilar physical structures. Seasonal variability follows closely seasonal variations in chemical composition of the butterfat. The June oiling-off maxima and October-November minima occur simultaneously with the minimum and maximum hardness of butter manufactured in this region *(6)*.

Influence of gas content. There is a major difference in the gas contents of continuous and of conventional Alberta butters. The former ordinarily contains no gas, or at least only a negligible amount, whereas the latter has a gas content of 1.5-3.5%. In order to determine the influence of gas content on oiling-off, butters of the continuous type were manufactured in the experimental continuous butter-making machine developed in this Department *(15)*. Nitrogen gas was incorporated into these butters in various concentrations (Table 1) and the oiling-off tendencies of the butters were measured. It is evident that oiling-off was not significantly influenced by even high concentrations of incorporated gas. This does not confirm the view of Mohr and Baur *(7)* that a high air content increases oiling-off. Pedersen *(9)* found that the application of vacuum during the working of butter did not influence oiling-off.

Continuously made butter characteristically lacks the stickiness of conventional butter. During these experiments the incidental, but interesting, observation was made that stickiness of the continuous type butter increased with

TABLE 1

Oiling-off of continuous type butter at 25° C. as influenced by the gas content

Butter	Gas content	Oiling-off
(No.)	*(%)*	*(%)*
1	0	17.7
1	1	18.2
1	18	18.6
2	1	19.4
2	4	20.6
2	17	20.6
3	0	17.7
3	1	17.3
3	7	17.0

increasing gas content. Differing gas contents may be the explanation of the difference in stickiness between continuous and conventional butters.

Influence of temperature. Oiling-off of both continuous and conventional butters at 28° C. was also studied, but the general picture remained unchanged. Seasonal variation followed the same pattern as that found at the lower temperature, but extended through the ranges 14.4-25.1% and 10.4-20.1% for continuous and conventional butters, respectively. There was, therefore, increased oiling-off in both types of butter and the average difference between the two types, 3.5%, was higher at this temperature.

Influence of sample size and shape. Butter plugs of varying sizes and shapes were tested to determine the influence on oiling-off. From the results (Table 2),

TABLE 2

Oiling-off at 25° C. as influenced by the size and shape of the test sample

Butter		Contact surface			
No.	Type	Shape	Cross section	Height	Oiling-off
			(cm.²)	*(cm.)*	*(%)*
1	G.F.	Square	6.25	2.5	12.7
1	G.F.	Square	6.25	5.0	7.6
1	G.F.	Rectangular	12.50	2.5	12.3
2	G.F.	Square	6.25	2.5	12.9
2	G.F.	Circular	10.54	2.5	13.0
3	Conv.	Square	6.25	2.5	11.7
3	Conv.	Square	6.25	5.0	6.9
3	Conv.	Circular	10.54	2.5	11.4

it is to be observed that oiling-off, when expressed as a percentage of the weight of the original test sample, is a function only of the height of the sample and is not related to the area in contact with the paper.

Influence of filter paper type and size. Determinations of oiling-off were made using different grades and sizes of paper, i.e., coarse (Whatman No. 4), medium (Whatman No. 1), fine (Whatman No. 5), and Whatman No. 1 with diameters of 9, 11, 12.5, and 15 cm.

From the results (Table 3), it appears that oiling-off on fine filter paper is lower than on medium or coarse paper, between which latter grades there is no

TABLE 3

Oiling-off at 25° C. as influenced by grade and size of filter paper

Filter paper		Surface area 1 circle	Oiling-off, %	
Diameter	Grade		Conventional butter	Continuous butter
(cm.)		*(cm.²)*		
12½	Fine	123	8.5	9.8
12½	Medium	123	9.6	13.0
12½	Coarse	123	9.2	12.7
9	Medium	64	9.2	11.5
11	Medium	95	9.3	12.0
12½	Medium	123	9.8	13.3
15	Medium	141	10.2	14.1

significant difference. Oiling-off also increases with increasing surface area of the filter paper.

DISCUSSION

As the physical structure of continuously made butter differs considerably from that of conventional butter, dissimilar oiling-off properties are to be expected. Indeed, the general observation has been made that the former type has a more oily appearance than the latter type of butter. The oiling-off tendency is an indication of the amount of free liquid fat in the butter, as well as of the character of the structure binding the liquid portion of the fat. Continuous butter has a much coarser crystalline structure than conventional butter *(14)*, which suggests easier drainage of the butter oil from the continuously made product. Moreover, since a considerable portion of the liquid fat in conventional butter is confined within the fat globules *(4, 8)*, a much smaller proportion can be expected to be available for drainage than with continuous butter. The difference in oiling-off between the two types of butter was fairly constant throughout the year.

Oiling-off properties may be expected to vary with the composition of the butterfat, which is subject to considerable seasonal change. According to Hilditch *(3)*, the glycerides in butterfat are of three general types, viz., (1) trisaturated, (2) monounsaturated-disaturated, and (3) diunsaturated-monosaturated. Seasonal differences in the composition of butterfat involve mainly Types 1 and 3, whereas Type 2 remains fairly constant. As Type 3 glycerides are those with the lowest melting point, it is not surprising that the seasonal variations in oiling-off were as pronounced as was demonstrated.

Since oiling-off fluctuates with such variables as temperature, height of the butter block, grade and size of filter paper, etc., the results of some of the investigations reported in the literature can not be compared closely with each other or with those reported herein. If it becomes desirable to make such comparisons, it will be necessary for investigators to use a standardized procedure.

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RELATIONSHIP BETWEEN MELTING QUALITY AND HARDNESS OF CHEDDAR CHEESE[1]

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SUMMARY

The relationship between melting quality, expressed as melting time, and cheese hardness was investigated. A relatively high correlation coefficient, +0.799, was found. Cheese hardness can be used as a means of predicting the melting quality of Cheddar cheese. The relationship of the fat content, the moisture content, and pH values to the melting quality and hardness of Cheddar cheese was investigated. These factors influenced hardness more than melting quality, but the effect, either separately or combined, was not strong enough to account for the wide variations found in melting quality or hardness.

Cheddar cheese is melted in the preparation and processing of many foods, both in industry and in the home. The term melting quality has been used to denote the ease with which cheese melts when subjected to heat. However, the term does not mean a single physical property but a combination of several, such as the time required for melting, the appearance of the melted cheese, i.e., smooth or stringy, and the amount of free fat separation. The combined effect of such factors should be considered for the specific purpose for which the cheese is melted. Thus, a cheese that has a satisfactory melting quality for one purpose may not have it for another purpose.

Study of the melting quality of cheese has been hindered by the lack of a suitable method for measuring melting quality, and the inability to predict this quality for cheese. As far as can be determined, there are no reports in the literature relating specifically to the melting properties of Cheddar cheese. Also, the available literature pertaining to the melting properties of process cheese is meager, the only information being a series of unpublished experiments *(2)* and a study by Arnott *et al. (1)* which included Cheddar cheese. Methods for evaluating the melting properties of cheese consisted either of observing standard-sized cheese samples after they had been heated over a steam bath or an electric heater for a given time *(2)*, or of measuring the height of a cheese sample before and after a heat treatment, at a constant temperature for a given time *(1)*. In part, the object of the work reported here was to attempt to correlate melting quality with certain other cheese properties, such as cheese hardness, that may be used as a means of predicting the melting quality of Cheddar cheese.

EXPERIMENTAL PROCEDURE

General. Cheddar cheese was obtained from a commercial process-cheese manufacturer. The samples were selected at random from various lots of current

Received for publication November 12, 1957.

[1] The data in this paper are from a thesis presented by R. W. Weik in partial fulfillment of the requirements for the degree of M.S., University of Minnesota, Scientific Journal Series Paper No. 3856, Minnesota Agricultural Experiment Station.

and aged cheese manufactured from pasteurized milk in Minnesota, Wisconsin, Iowa, and Michigan.

A special device of stainless steel construction (Figure 1) was designed to obtain cheese samples measuring 2.25 by 2.25 by 4.50 in. Samples were taken

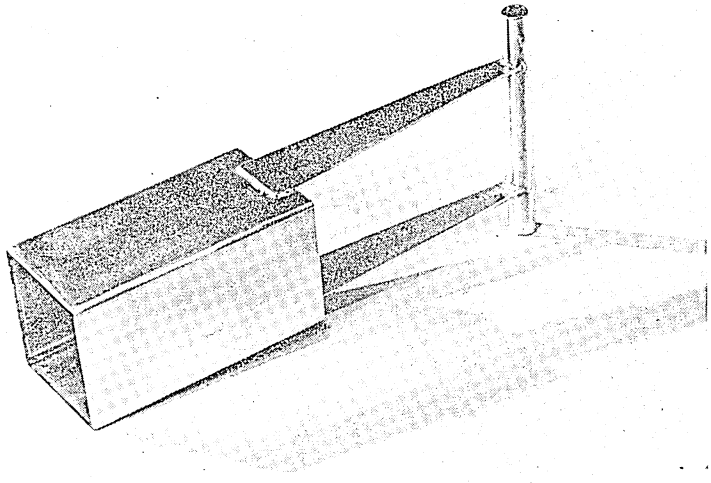


FIG. 1. Cheese-sampling device.

from each cheese in a position about one-half the distance from the center to the outer edge of the cheese.

The fat and moisture analyses were made following the method described by the Dairy Division of the Agricultural Marketing Service, USDA *(5)*. The pH values were determined as described by Arnott *et al. (1)*.

Melting quality. There are many immeasurable factors involved in the overall melting quality of cheese. Therefore, the time required for melting, an important factor, was selected as a means of evaluating melting quality. The melting time was considered an index of melting quality; a short melting time was indicative of a good melting quality, whereas a long melting time suggested a poor melting quality.

Details of the method are as follows: All cheese samples were placed in a refrigerated room, maintained at 50° F. ± 2° F., for 24 hr. prior to melting-time determinations. From the cheese sample mentioned above a standard cylinder of cheese was obtained, following the method of Arnott *et al. (1)*. The cheese cylinder was placed in an 8-in. test-tube which was closed with a cork stopper. The test-tubes containing the samples were placed in a visibility water bath[2] maintained at 80° C. ± 1° C., and the time, in seconds, required for the sample to melt was determined. The criterion for complete melting of the sample

[2] Manufactured by Blue M Electric Company, Blue Island, Illinois.

was the moment that complete loss of the original shape of the sample became evident. This method also permitted observation of differences in appearance of the cheese during melting.

Hardness. Cheese hardness was determined with the Cherry-Burrell Curd Tension Meter as modified by Irvine *(4)*. The 500-g. capacity scale supplied with the instrument was too small and was replaced with one of 1,000-g. capacity. All determinations were made in a refrigerated room maintained at 50° F. ± 2° F. Hardness was expressed as the maximum force, in grams, required to force the wire-cutter through the cheese.

RESULTS AND DISCUSSION

Melting quality. Although most of the cheese samples would melt, considerable variation was noted in the time required for melting, as well as in the appearance of the melted cheese. The majority of samples melted between 290 and 600 sec.; however, a few samples required a longer time. For example, two samples were heated for 3,180 sec. During this time no appreciable change in shape was observed.

One thousand seconds was arbitrarily selected as a practical limit for heating, since samples not melting in this length of time rarely showed any marked change in shape and did not melt completely. Samples exceeding 1,000 sec. were not included in the calculations of correlation coefficients.

Generally, the melted cheese samples requiring the least heat treatment were soft and easily stirred. The melted cheese exhibited definite plastic properties. Somewhat the reverse was observed for samples requiring melting times of 500 sec. and greater. Although melted, these samples were difficult to stir and generally quite elastic. Free fat was present, but in much smaller amounts than from the easily melted cheese.

Hardness. Theoretically, to measure cheese hardness, only the force required to break the cheese surface would have to be known. This force is the yield value or yield point. Since the yield value was difficult to determine, the hardness was taken as the maximum force required to push the wire through the cheese. Most of the cheese exhibited a hardness of less than 500 g. The range of hardness in samples was from 270 to 756 g.

The data that follow, from 89 samples of cheese, were analyzed following the method of Friedman and Foote *(3)*.

Relationship between melting quality and hardness. This relationship was considered to determine whether the just-described method of evaluating cheese hardness could be used to predict the melting quality of Cheddar cheese, expressed in terms of melting time. The relationship is shown (Figure 2). The extent of relationship is indicated by the scatter of points around the regression line and the relatively high correlation coefficient of +0.799. An inspection of the plot shows that the points are grouped fairly close around the regression line up to a hardness of about 500 g. This grouping includes approximately 65% of the samples.

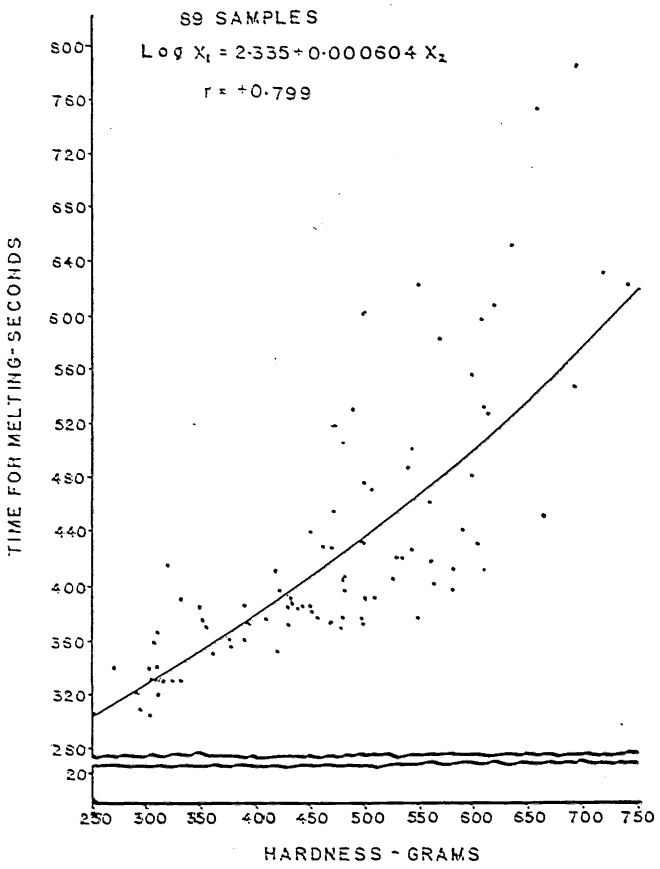


FIG. 2. Relationship of melting time to hardness of Cheddar cheese.

The coefficient of determination for this relationship was 0.638. This was interpreted to mean that the evidence presented indicated that 63.8% of the variability in melting time was associated with cheese hardness. Thus, it would appear that the relationship was high enough to use cheese hardness as a means of predicting melting quality. The degree of predictability was higher below the 500-g. level, but the trend of a longer melting time with an increase in hardness continued above the 500-g. level.

Since the coefficient of determination indicated that 63.8% of the variability in melting time was associated with cheese hardness, the remaining 36.2% of the variability must be associated with another factor, or combination of factors. On the assumption that certain constituents or characteristics of cheese would have an effect on its melting quality and hardness, the influence of the fat content, moisture content, and pH value on these characteristics was determined.

Effect of fat content, moisture content, and pH value on melting quality. The effect of these factors on melting quality was determined separately, followed by a determination of their multiple effect. The simple and partial correlation coefficients are presented (Table 1).

The effect of the fat content on melting quality, $r = +0.133$, has no statisti-

TABLE 1
Influence of various factors on the melting quality (X_1) of Cheddar cheese

Factor	Correlation coefficients	
	Simple	Partial
Fat content *(%)*—X_2	r12 = +0.133	r12.34 = a [b]
Moisture content *(%)*—X_3	r13 = −0.118	r13.24 = a
pH value—X_4	r14 = +0.234 [a]	r14.23 = +0.103

[a] Significant at the 0.05 level.
[b] a = Less than 0.001.

cal significance and is too low for any practical use in predicting the melting quality of cheese. The results are of the same magnitude as those reported by Arnott *et al. (1),* who used a different method for evaluating melting quality.

The lack of relationship between the moisture content and melting quality is shown by the correlation coefficient of −0.118, which confirms the results of Arnott *et al. (1).* An insignificant value, statistically, this value is also too low to warrant use of the moisture content as a means of predicting the melting quality.

The relationship of pH to melting quality is indicated by a correlation coefficient of +0.234. Although statistically significant, this value does not indicate a degree of relationship strong enough to use the pH value alone as a means of predicting the melting quality of Cheddar cheese.

The partial correlation coefficients were obtained, to compare the simple correlation coefficients just presented with the net effect of each one of the three factors on the melting quality, after the full effect of the two other factors was considered.

The effect of the fat content on melting quality (r 12.34) was not significantly different from zero when the influence of the moisture content and pH value was considered. A similar result was obtained for the relationship between the moisture content and melting quality (r 13.24) when the effect of the two other factors was considered. A value of +0.103 was obtained for the influence of pH on melting quality (r 14.23) when the effect of the fat and moisture content was considered. It will be remembered that the values presented for the simple correlation coefficients were small and of no practical importance regarding the predictability of melting quality. The partial correlation analysis presents no further evidence that would indicate a stronger relationship between any of the factors and melting quality. However, the values, r 14 = +0.234 and r 14.23 = +0.103, support the conclusion of Eckberg and Mykleby *(2)* that a low-pH cheese showed a tendency toward better melting quality, and a high-pH cheese a tendency toward poor melting quality.

Since a slight relationship was found between melting quality and fat content, moisture content, and pH value, the multiple effect of these three factors together on melting quality was determined. The regression equation was calculated to be:

$$\log X_1 = 1.691 + 0.0105X_2 + 0.00206X_3 + 0.0919X_4$$

where X_1 represents melting time, X_2 represents the fat content, X_3 represents

the moisture content, and X_4 represents the pH value. This equation indicates that the logarithm of the melting time and, therefore, the melting time, would increase with an increase in all factors considered. However, the degree to which variations in the melting quality were related to the combined action of the fat content, moisture content, and pH value was small, the coefficient of multiple correlation being only 0.220. This analysis indicates that although the combined influence of the pH value, fat and moisture contents influences the melting quality more than the separate effect of the fat and moisture contents, the combined influence of these three factors is not sufficient for use in predicting the melting quality of cheese.

Effect of the fat content, moisture content, and pH values on cheese hardness. The effect of these three factors on cheese hardness was considered in a manner similar to that for melting quality. The simple and partial correlation coefficients are shown (Table 2).

TABLE 2

Influence of various factors on the hardness (X_1) of Cheddar cheese

Factor	Correlation coefficients	
	Simple	Partial
Fat content *(%)*—X_2	r12 = +0.349 [b]	r12.34 = +0.181
Moisture content *(%)*—X_3	r13 = −0.347 [b]	r13.24 = a [c]
pH value—X_4	r14 = +0.245 [a]	r14.23 = a

[a] Significant at the 0.05 level.
[b] Significant at the 0.01 level.
[c] a = Less than 0.001.

The relationship of the fat content to cheese hardness is indicated by a correlation coefficient of +0.349. Although highly significant statistically, this value indicates that approximately 12% of the variation in hardness is associated with the fat content. Thus, the fat content exerts little influence on cheese hardness.

Similar results were obtained for the relationship between hardness and the moisture content. The correlation coefficient of −0.347, although highly significant, indicates that approximately 12% of the variation in hardness is associated with the moisture content.

The effect of the pH value on cheese hardness is indicated by the correlation coefficient +0.245. Despite this statistically significant value, the pH of cheese exerts little influence on cheese hardness, only 6% of the variability in hardness being associated with the pH value.

Partial correlation coefficients were calculated to provide more information concerning the effect of the pH value and fat and moisture contents on cheese hardness. A value of +0.181 was obtained for the influence of the fat content on hardness (r 12.34) when the effect of the moisture content and pH value was considered. When the single influence of the fat content on hardness was considered, the correlation coefficient was +0.349. The apparent effect of the fat content is then markedly reduced (r 12.34 = +0.181) when the influence of the moisture content and pH value is considered.

The influence of the moisture contents on hardness (r 13.24) was not significantly different from zero, when the effect of the fat content and pH value was considered. A similar result was obtained for the influence of the pH value on hardness (r 14.23) when the effect of the fat and moisture contents was considered.

This analysis presents no further evidence that would indicate a stronger relationship between any of the factors and cheese hardness. However, since the correlation coefficients were either highly significant, or nearly so, and the majority of cheese exhibited a hardness of less than 500 g., the multiple effect of these three factors on cheese hardness was determined. The regression equation was calculated to be:

$$X_1 = 207.774 + 27.373X_2 - 27.328X_3 + 60.121X_4$$

where X_1 represents cheese hardness, X_2 represents the fat content, X_3 represents the moisture content, and X_4 represents the pH values. This equation indicates that hardness increased with an increase in fat content and pH value and with a decrease in moisture content. The coefficient of multiple correlation was found to be 0.409. This value, highly significant statistically, indicates that approximately 16% of the variations in cheese hardness was associated with the combined influence of the fat and moisture contents and pH values.

Although the pH values and fat and moisture contents have been shown to influence cheese hardness more than they do melting quality, the effect of these three factors, separately or combined, on cheese hardness is not strong enough to account for the wide variations found in cheese hardness.

There are many factors not included here that undoubtedly influence both melting quality and cheese hardness. Although not a part of this study, variations in cheese structure may have a great influence on both hardness and melting characteristics. This may be one of the unexplained variables mentioned in relation to melting quality and hardness.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Land O' Lakes Creameries, Inc., Minneapolis, Minnesota, for their assistance in supplying the Cheddar cheese samples, and to Mrs. Martha Hilgeman for technical assistance.

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FACTORS AFFECTING TERRAMYCIN ACTIVITY IN MILK, BROTH, BUFFER, AND WATER[1]

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SUMMARY

Studies were conducted to determine the effect of heat, storage, total solids content, bacterial metabolism, and various gases upon the degree of terramycin inactivation in milk, broth, buffer, and water. Terramycin activity was determined by a modified disk assay technique and the acid inhibition method. The rate of heat inactivation of the antibiotic at 143 and 160° F. for 50 min. followed the first-order reaction in milk and buffer but not in water. When autoclaved at 15 lb., the antibiotic was inactivated completely within 5 min. in all three media. The *Z* values for complete thermal inactivation curves for terramycin, aureomycin, streptomycin, and penicillin in milk were 48, 51.5, 36, and 49, respectively. Terramycin was more heat-susceptible than the three other antibiotics. Heat inactivation of terramycin in reconstituted milk of different solids concentrations was inversely related to the solids content. During storage terramycin lost its potency at a faster rate in the unheated than in the heated system. This may have been owing to natural microorganisms and their enzymes in the unheated media, because it was observed that the loss of terramycin potency in a broth inoculated with a raw milk bacterial suspension was directly related to bacterial multiplication. When sterile milk containing terramycin was exposed constantly to nitrogen, oxygen, or air, the antibiotic lost its potency, the greatest loss occurring with oxygen.

Terramycin (oxytetracycline), an analogue of the tetracycline antibiotics, has been shown to possess a marked effect against various microorganisms, including gram-positive, gram-negative, aerobic, and anaerobic bacteria and many viruses *(5, 6)*. When used as a therapeutic agent, it has been shown also to possess a low degree of toxicity *(4)*. It is, therefore, used singly or in combination with other antibiotics, for the treatment of various diseases. Cromley and Hagely *(3)* and Simon and Schmidt *(15)* have reported terramycin to be therapeutically effective against staphylococcic and streptococcic mastitis. Schipper and Petersen *(10)* have shown that following intravenous and intramammary administration terramycin appears in milk, blood, and urine.

Regna and Solomons *(9)* have reported that terramycin compares favorably with the more stable antibiotics now in common use. Previous antibiotic studies conducted at this laboratory have shown that plant practices like heat treatment and storage inactivate penicillin *(13)*, streptomycin *(12)*, and aureomycin *(11)*. Information relative to the effect of heat, storage, milk solids, bacterial metabolism, and various gases upon the degree of terramycin inactivation has been obtained and is presented.

Received for publication August 5, 1957.

[1] Article No. 9:57 of the Department of Dairy Technology, The Ohio State University. Supported by a grant from the U. S. Public Health Service (National Institutes of Health) and by The Ohio Dairy Products Research Fund.

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METHODS

Throughout this study, the terramycin activity was determined by the disk assay method and the acid inhibition method. In the disk assay method, an enriched agar was used as the assay medium and *Bacillus cereus* as the test organism. The enriched agar consisted of the following gram-portions per 1,000 ml: yeast extract, 5, lactose, 5, dipotassium phosphate, 5, neopeptone, 2.5, peptonized milk, 2.5, and agar, 15. The medium was adjusted to pH 6.8 before sterilization. The principle, the details of the disk assay method, and the technique of heating and storage have been described earlier *(11, 13)*. In all the phases of this study the antibiotic was added to milk, buffer, water, or broth in concentrations ranging 0.3 and 3.2 γ per millilter.

RESULTS AND DISCUSSION

Effect of various heat treatments on terramycin. The initial phase of this study was to determine the effect of various heat treatments upon terramycin in milk, 0.1 *M* potassium phosphate buffer (pH 4.7), and distilled water. Terramycin was added to the milk, phosphate buffer, and water at the rate of 0.84 to 1.0 γ per milliliter and heated at 143 and 160° F. The rates of inactivation of the antibiotic were determined following 10, 20, 30, 40, and 50 min. of heating. The data obtained are recorded (Figure 1). The following differential equation proposed by Prutton and Maron *(8)* was used in order to determine whether or not the rate of terramycin heat inactivation against time was of the first order:

$$\text{Log } (a-x)=\frac{-k_1}{2.303}\,t + \log a$$

Where a = initial concentration, x = loss, t = time, and k is a constant.

In Figure 1, the logarithm of the per cent of residual terramycin has been plotted on the ordinate and the time in minutes on the abscissa. The loss of the antibiotic was found to be related directly to the heat treatment. The rate of terramycin inactivation owing to heat was faster in water than in buffer or milk. In general, these results are in close agreement with those of Bohonos *et al. (2)*, who have reported that during heating, aureomycin, terramycin, and tetracycline are less labile at a lower pH than under neutral or alkaline conditions.

It was observed that the rate of heat inactivation of terramycin at 143 and 160° F. for 50 min. in milk and buffer followed the first-order reaction, because a straight-line curve was obtained when the logarithm of the concentration of the residual antibiotic was plotted against time. In water, the rate of inactivation followed the first-order reaction at 143° F. When heated at 160° F., the inactivation rate of terramycin followed the first-order reaction up to the first 30 min. of heating only, following which the inactivation rate was progressively faster and resulted in a complete loss of the antibiotic activity in 50 min.

Studies were also conducted to determine the rate of terramycin inactivation when heated at 15 lb. (approximately 250° F.) in an autoclave. An empty autoclave was heated with steam to remove air, quickly opened, the samples inserted,

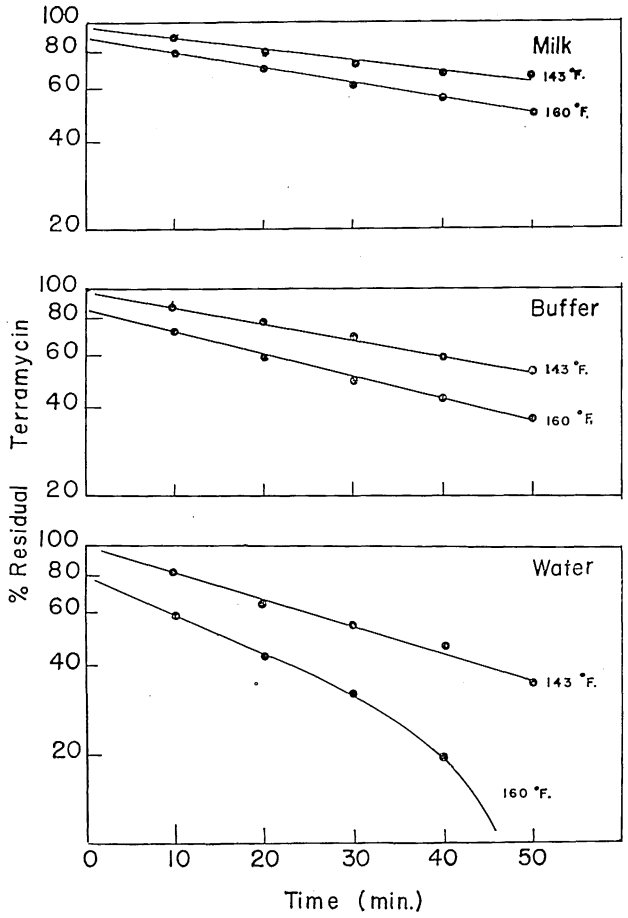


FIG. 1. Thermal inactivation of terramycin in milk, buffer, and water at 143 and 160° F.

and pressure increased quickly. As soon as the pressure reached 15 lb., the time was recorded. At the end of the desired period the pressure was reduced rapidly and the samples were withdrawn and cooled in an ice-bath. Milk, buffer, and water samples, inoculated with known amounts of terramycin, were subjected to the above treatment for 5, 10, and 15 min. and assayed for the antibiotic activity. Separate lots of the test samples were used for each time interval. It was observed that under the conditions of the above experimental procedure the antibiotic lost its potency completely within 5 min. in all three media.

Complete heat inactivation of terramycin in milk. The next phase of this study was the determination of the time-temperature relationships necessary for the complete inactivation of small concentrations of terramycin in milk. Fresh, raw, mixed-herd milk was obtained from the University dairy, and terramycin was added at the rate of 0.5 to 0.55 γ per milliliter and heated at various temperatures until the antibiotic potency was lost completely. The assay procedure included the disk assay method and also the starter activity method. As used in the previous studies *(11)*, the nondevelopment of a zone of inhibition by the first

method and no retardation in the acid development in the latter method were taken as criteria for the complete inactivation of the antibiotic. Average results of from three to six trials conducted at each temperature have been presented (Figure 2). The temperature of heating is plotted on the abscissa and the logarithm of time required for complete inactivation is plotted on the ordinate. To compare the heat stability of terramycin with that for other antibiotics, the thermal inactivation curves for penicillin, streptomycin, and aureomycin reported previously also have been included in the same figure.

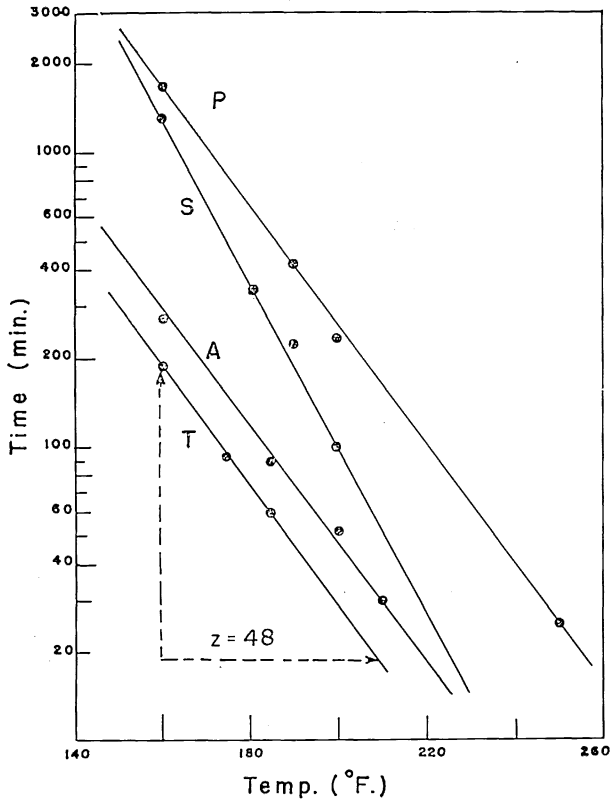


FIG. 2. Complete thermal inactivation of various antibiotics in milk. P=Penicillin, S=Streptomycin, A=Aureomycin, T=Terramycin.

Terramycin was inactivated completely in milk when heated at 160° F. for 190 min., at 175° F. for 92 min., or at 185° F. for 60 min. A linear relationship was observed between the logarithm of time and the temperature, and by using the formula proposed by Ball *(1)* the Z value for the thermal inactivation curve for terramycin was calculated to be 48.

The data revealed that terramycin was more susceptible to heat than were the three other antibiotics. It was observed that when heating at 160° F., 190 min. were required to inactivate terramycin, compared to 280 for aureomycin, 1,320 for streptomycin, and 1,705 for penicillin. Compared to the values of 51.5, 36,

and 49 for the thermal inactivation curves for aureomycin, streptomycin, and penicillin, respectively, the Z value for terramycin was 48.

Effect of milk solids upon heat inactivation of terramycin. To determine whether or not the solids content of milk was the factor responsible for the antibiotics being more heat-labile in water and buffer than in milk, a study was conducted to determine the rate of heat inactivation of terramycin in milk of various solids concentrations. Using skimmilk powder, reconstituted milk was prepared containing 10 and 25% total solids. Terramycin was added to the milk at the rate of 0.71 and 1.06 γ per milliliter, and loss of antibiotic potency was determined following heating at 160 and 185° F. for 30 min. The data obtained are presented (Table 1). Upon heating at 160° F. for 30 min., 26% of the anti-

TABLE 1

Effect of heat on terramycin in milk containing 10 and 25% total solids

	10% T.S. Milk				25% T.S. Milk			
	Heated at 160° F. for 30 min.		Heated at 185° F. for 30 min.		Heated at 160° F. for 30 min.		Heated at 185° F. for 30 min.	
Antibiotic inoculated	Antibiotic content	Loss	Antibiotic content	Loss	Antibiotic content	Loss	Antibiotic content	Loss
(γ/ml.)		*(%)*	*(γ/ml.)*	*(%)*	*(γ/ml.)*	*(%)*	*(γ/ml.)*	*(%)*
0.71	0.58	18.3	0.30	57.7	0.59	16.9	0.28	60.5
0.71	0.57	19.7	0.31	56.3	0.60	15.5	0.30	57.7
1.06	0.72	32.1	0.30	70.7	0.90	15.1	0.47	55.7
1.06	0.70	33.9	0.34	67.9	0.88	17.0	0.46	56.6
Average loss		26.0		63.1		16.1		57.6

biotic was lost in milk with 10% solids, compared to a 16% antibiotic loss in milk containing 25% solids. A similar trend was obtained when the samples were heated at 185° F. In reconstituted milk with 10% total solids there appeared to exist a direct relationship between the terramycin content of the samples and the extent of heat inactivation of the antibiotic. However, this was not true in the 25% total solids milk.

Attempts were made to determine the loss of antibiotic activity in 40% solids milk, also. However, the samples could not be assayed accurately, because the milk became too viscous upon heat treatment and could not be pipetted. Data revealed that heat inactivation of terramycin in reconstituted milk of different solids concentrations was inversely related to the solids content, indicating that milk solids perhaps exert a protective effect upon the antibiotic during heating.

Effect of storage upon terramycin in raw and heated samples. To determine the effect of storage upon terramycin in raw and heated milk, phosphate buffer, and water, the samples were inoculated with the antibiotic at the rate of 0.32 to 3.22 γ per milliliter. Using the same experimental procedure described in previous reports *(10, 11)*, the milk, buffer, and water samples were heated at 143 and 160° F. for 30 min. The raw and processed samples were then stored at from 2 to 4° C. and assayed every week for 5 wk. The per cent losses of terra-

mycin in the three media upon heating and storage are presented (Table 2). These data represent average results of from two to six trials.

Similarly to what was observed in a previous experiment (Figure 1), the heat inactivation of the antibiotic was found to be related directly to the extent of heat applied, and the rate of loss of terramycin in milk, buffer, and water was in an increasing order. There were considerable variations between the results obtained in various trials. As observed in previous studies *(11–13)*, there was no apparent relationship between the concentration of the antibiotic and the percentage loss of terramycin upon heating.

TABLE 2

Per cent loss of terramycin potency in milk, buffer, and water owing to heat treatment and storage

Heat treatment	No. of trials	Terramycin inoculated	Loss upon heating	Cumulative loss upon storage				
				1 wk.	2 wk.	3 wk.	4 wk.	5 wk.
		(γ/ml.)		*(%)*				
			Milk					
Raw	6	0.32-1.29	.....	13.9	21.8	28.2	33.0	41.9
143° F. for 30 min.	3	0.32-0.90	23.6	5.8	8.6	10.8	12.5	20.2
160° F. for 30 min.	3	0.40-1.29	35.6	1.5	6.4	.....	12.8	16.3
			Buffer					
Unheated	6	0.43-3.22	.....	18.0	21.4	25.8	28.9	33.9
143° F. for 30 min.	3	1.00-3.22	32.4	12.3	14.1	16.9	17.4	24.7
160° F. for 30 min.	2	0.43-1.28	48.4	.....	.....	15.2	21.2	22.6
			Water					
Unheated	6	0.43-3.22	.....	19.3	32.1	34.4	39.0	48.9
143° F. for 30 min.	3	1.00-3.22	41.4	4.7	10.6	12.7	19.0	25.1
160° F. for 30 min.	3	0.43-1.28	66.4	.....	13.1	.....	25.7	27.3

It was observed that during storage terramycin lost its potency at a faster rate in the unheated samples than in the heated ones. Similar results were obtained with penicillin, streptomycin, and aureomycin in previous studies. In the milk group, during storage for 5 wk., an average of 41.9% of terramycin was lost in the raw samples, compared to 20.2% in the samples that had been heated at 143° F. and to 16.3% in the samples heated at 160° F. Essentially similar trends were observed in the buffer and water samples. Under the conditions of this study there was no relationship between the terramycin content and the loss of its activity during storage. The fact that the antibiotic acivity decreased during storage is in contrast to the reports of Regna and Solomons *(9)*, who observed that an aqueous solution of terramycin hydrochloride at pH 3.0–9.0 did not show detectable loss in activity during storage at 5° C. for one month.

In another study relative to the preservation of milk with antibiotics *(14)*, it was observed that during storage raw milk samples inoculated with terramycin, aureomycin, streptomycin, or penicillin developed putrefaction, proteolysis, and microbial deterioration in from ten to 14 days. In milk inoculated with terramycin or aureomycin and then pasteurized, no microbial spoilage was observed for from 3 to 4 wk. Although in the present study no attempts were made to determine bacterial numbers in the raw and heated milk, on the basis of the

study just mentioned *(14)* it may be hypothesized that during storage there was greater bacterial metabolism in raw milk than in heated milk.

Effect of bacterial metabolism on terramycin activity. Because the inactivation of terramycin during storage was faster in raw milk than in heated milk, studies were made to determine the effect of the microorganisms present in raw milk upon the antibiotic activity.

Raw milk was inoculated with terramycin and held at three temperature ranges—at 2–4, 22–26, and 35° C. Terramycin activity was determined following one, two, and three days of storage. The samples, held at from 2 to 4° C., did not show any appreciable loss of antibiotic activity. The loss of antibiotic activity could not be determined in the samples held at the higher temperatures, because the samples could not be assayed, owing to coagulation of milk. Attempts were made, therefore, to determine the effect of the microorganisms present in raw milk upon the antibiotic in enriched broth.

A raw milk bacterial suspension was prepared by inoculating raw milk in the enriched broth (composition the same as used in the enriched agar, except for agar) and incubating it at 30° C. for from 14 to 16 hr. The broth culture was transferred daily in fresh broth and was used in these trials after it had been transferred from six to seven times. Two 100-ml. lots of enriched broth containing 0.60-0.62 γ of terramycin per milliliter were inoculated with the broth suspension of raw milk bacteria at the rate of 1 and 5%, respectively. Each lot was divided into two parts. One part of each lot was incubated at 35° C. and the other part was held at 2° C. in a refrigerator. The antibiotic content and total bacterial counts were determined immediately and following one, two, and three days of incubation. The samples were stored for a maximum of three days, in contrast to 5 wk. in the immediately preceding phase of the work (Table 2), because the preliminary trials revealed that the antibiotic loss was considerably faster in the broth inoculated with a bacterial suspension than in the unheated milk, buffer, or water. Also, because the medium composition and incubation temperature influence the type and numbers of bacteria in a medium, the present phase of work was conducted not to duplicate but to investigate what might happen in raw milk.

Average results of from two to three trials are presented (Figure 3). It was observed that during holding at both the temperatures there was an increase in the bacterial count and a concomitant decrease in the antibiotic activity. As should be expected, there was greater bacterial multiplication at 35 than at 2° C. The rate and amount of the antibiotic loss were greater in the samples inoculated with 5% suspension or incubated at 35° C. than in the samples inoculated with 1% suspension or incubated at 2° C. The results clearly indicate that the loss of the antibiotic potency was directly related to the extent of bacterial metabolism. The present results are in close agreement with observations made in a previous study *(12)*, in which it was observed that in 2 wk. there occurred an appreciable loss of streptomycin activity in milk stored raw, whereas no apparent loss of the antibiotic occurred in sterilized milk. These results are not in complete agreement with the findings of Jurtshuk *et al. (7)*. They observed that zones

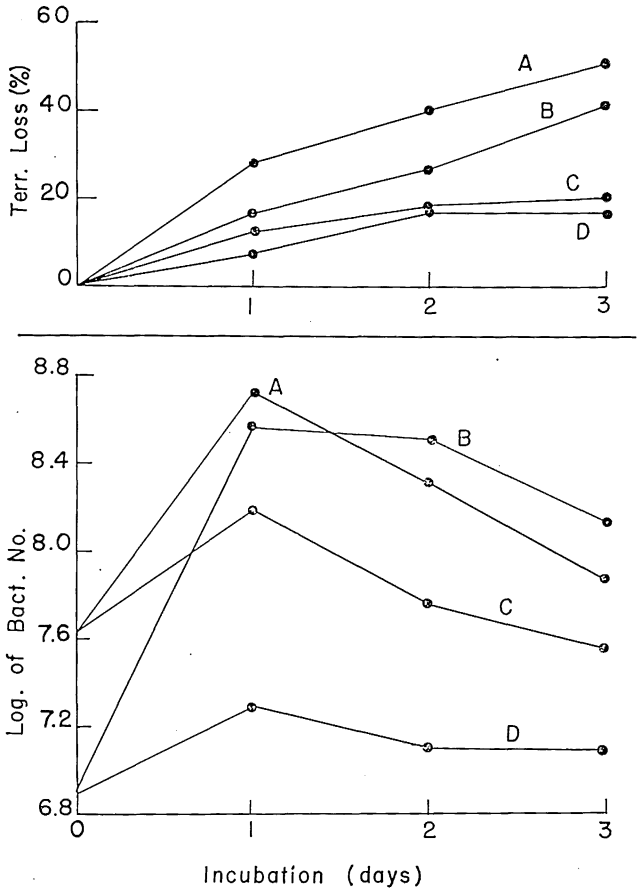


FIG. 3. Per cent loss of terramycin and rate of bacterial multiplication during incubation of broth inoculated with raw milk bacterial suspension. Inoculum: A=5%, B=1%, C=5%, D=1%, and incubation temperatures, 35, 35, 2, and 2° C., respectively.

of inhibition produced by residual aureomycin after incubation with mixed suspensions of bovine rumen bacteria were smaller than with aureomycin incubated with buffer. This was termed by them to be owing to some nonspecific type of inactivation. However, when similar work was conducted with terramycin, they found no appreciable differences between zone diameters obtained by terramycin incubated with buffer and terramycin incubated with rumen bacteria.

Effect of different gases upon terramycin activity in milk. In the search for causes and mechanisms involved in the inactivation of antibiotics, a study was conducted to determine the effect of various gases in air upon terramycin activity in milk. Twenty-five milliliter aliquots of fresh sterile milk were inoculated with from 0.96 to 1.1 γ terramycin per milliliter and dispensed in 25 × 200 mm. pyrex test-tubes with a side opening near the top of the tube. The tube was fitted with a rubber stopper with a narrow glass tubing running from the top to the bottom of the tube. Through the center glass tube, the milk was subjected to constant exposure to air, nitrogen, or oxygen by connecting the glass tubing to the

gas supply and constantly bubbling the gas through the milk system. The flow of the gas was controlled so as to produce about 50 to 60 bubbles per minute. All of these trials were conducted at room temperature (22 to 27° C.). An extra tube not containing the center tube and the side opening, and through which no gas was bubbled, served as a control. At the end of 4, 24, 36, and 48 hr. of exposure, aliquots of milk were withdrawn and assayed for antibiotic activity.

Average results of three trials are presented (Figure 4), which show the per cent loss of the antibiotic in milk upon constant exposure to various gases.

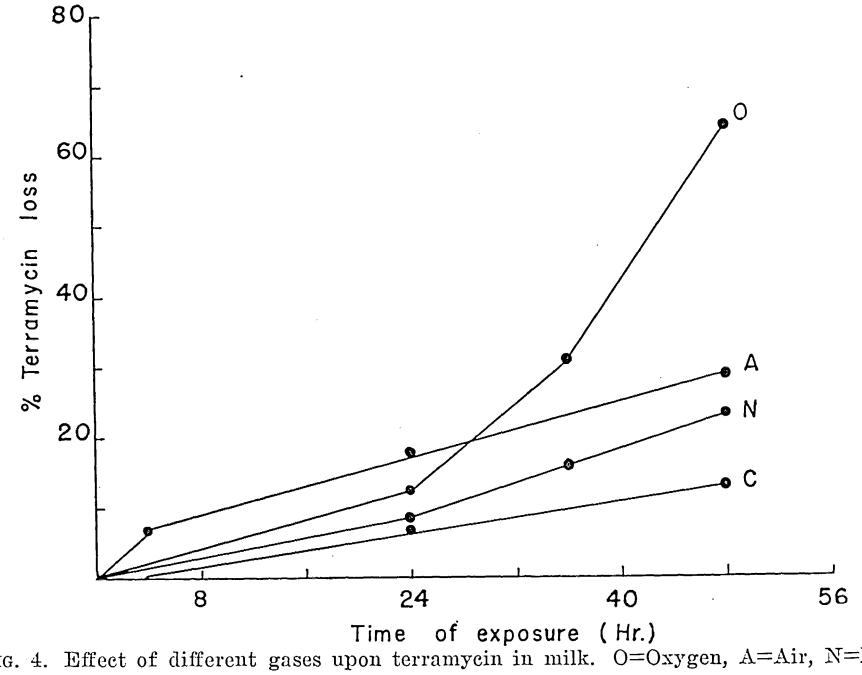


FIG. 4. Effect of different gases upon terramycin in milk. O=Oxygen, A=Air, N=Nitrogen, C=Control.

It was observed that the control samples not exposed to any gas lost about 13% of their antibiotic content in 48 hr. When milk containing terramycin was exposed to nitrogen, oxygen, or air, the samples lost part of their antibiotic activity and the loss was directly related to the time of exposure. Over a long period of exposure, oxygen was found to be more detrimental than air or nitrogen. At the end of 24 hr. of exposure to nitrogen, air, or oxygen, there resulted a loss of 8.4, 17.7, and 12.5%, respectively. Upon additional exposure of 24 hr., or at the end of a total exposure of 48 hr., 23.3% terramycin was lost in milk when exposed to nitrogen, 28.9% when exposed to air, and 64.5% when exposed to oxygen. The greater inactivation of the antibiotic when exposed to oxygen might be owing to oxidation of the antibiotic. The control milk samples in rubber-stoppered tubes, through which no gas was bubbled, also lost about 13% of their antibiotic content. These results suggest that the loss of antibiotic activity during storage might have been owing partly to the air present in the empty part of the tube. These observations may have significance to the dairy industry, because milk is often transported in cans or bulk tanks with air space.

CONCLUSIONS

Terramycin was found to be more heat-labile than penicillin, streptomycin, and aureomycin. The rate of the antibiotic loss was directly related to the extent of heat applied, and its heat-lability in milk, buffer, and water was in an increasing order. Heat inactivation of the antibiotic in reconstituted milk was inversely related to its total solids content. Storage loss of terramycin in milk might be owing partly to the presence of naturally present microorganisms and their enzymes in milk. Also, it is believed that the inactivation of the antibiotic during storage might be owing in part to oxidation.

ACKNOWLEDGMENTS

Sincere thanks are extended to Drs. I. A. Gould, H. H. Weiser, and W. L. Slatter for their interest and counsel in this study. Also, acknowledgment is made to Dr. R. C. Ottke, Chas. Pfizer and Co., Inc., for the generous supply of terramycin used in this study, and to Dr. H. L. Schoenlein of Difco Laboratories for supplying the *B. cereus* spore suspension used in part of this study.

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CONDUCTIVITY, PER CENT LACTOSE, AND FREEZING POINT OF MILK

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SUMMARY

Milk from individual cows and from groups of cows was examined for its freezing point and for factors affecting the freezing point.

Based on results obtained with 100 raw milk samples from individual cows, lactose was found to be the most important factor governing the freezing point of milk. Of the 62 samples with over 4.5% lactose, 23 (37%) had freezing points of −0.550° C. and below, and 6 (10%) had freezing points above the 3% water-tolerance level, namely, −0.5335° C.

Of the 38 samples with 4.5% lactose or less, none had a freezing point of −0.550° C. or below, but 22 (58%) had freezing points of above −0.5335° C.

The specific conductivity of milk varied inversely with the per cent lactose. Although in low-lactose mastitic milk a legal freezing point was observed, nonmastitic milk equally low in lactose showed freezing points above the legal minimum. Both prolonged storage of raw milk and pasteurization decreased the specific conductivity of milk and raised its freezing point.

Jackson and Rothera *(7)* have shown that lactose and the ionizable salts exert the greatest influence upon the osmotic pressure of milk, as measured by its freezing-point depression. They found that electric conductivity and per cent lactose showed a strict reciprocity, provided that the secretion of milk samples corresponded to the same period.

Coste and Shelbourne *(4)* observed that although there was no correlation between electric conductivity and per cent total solids, or between electric conductivity and total ash, there existed a marked correlation between electric conductivity and chlorine content of milk. These workers reached the conclusion that the osmotic pressure of milk was owing to the milk sugar and salts which it contained, and that an increase in the amount of lactose led to a decrease, not necessarily in the amount of salts but in the total number of their molecules and ions dissolved in milk. More recently, Barry and Rowland *(3)* have shown the inverse relationship between lactose and NaCl in milk.

In studying the effect of heat on milk, Magee and Harvey *(9)* observed that in fresh, raw milk about 26% of the total CaO was in diffusible form, as contrasted to only 20% in pasteurized milk and 15% in boiled milk. A significant reduction in Ca and Mg ions in pasteurized milk has also been reported by van Kreveld and van Minnen *(8)*.

The present work was undertaken to study further the ionic portion of milk, its concentration in different milks, and its influence upon the freezing point in raw and pasteurized milk.

Received for publication August 19, 1957.

EXPERIMENTAL PROCEDURE

Most milk samples reported on in this study were obtained from animals of the College herd. All samples were collected during the fall and winter months, in close succession for each portion of the study, and were obtained from evening milkings only.

Milk samples obtained from complete milkings of one or more cows were collected in glass milk bottles of quart size, previously rinsed with distilled water of low conductivity and allowed to dry. All samples were cooled immediately after being drawn, and kept in ice water until analyzed, with the exception of those samples analyzed while still warm. The latter samples were kept in a water bath at 37° C. for up to 2 hr. Conductivity readings and freezing-point determinations were made within 2 hr. after collection of samples, unless stated otherwise.

The degree of ionization of milk was measured in ohms of resistance at given temperatures and is reported in (mhos/cm)$\times 10^4$ of conductivity. The apparatus used consisted of a conductivity bridge (Industrial Instrument Co., Model RC-16B) with an accompanying conductivity cell. This cell was equipped with two permanently platinized electrodes and a standardized thermometer. The cell constant was determined with aqueous KCl solution of recommended concentration at the onset of the experiments. Periodic checks were made during the study to verify the constancy of the cell.

The freezing point of milk was determined by means of an improved, modified, official Hortvet cryoscope, having a mechanical stirrer. The A.O.A.C. method was observed both in the operation of the instrument and in the standardization of the thermometer *(2)*. Periodic checks were made to verify the constancy of the thermometer.

Lactose was determined by means of a Rudolph (Routine Model 60) double-field polarimeter, having a vernier reading of 0.05°. An electric sodium lamp supplied the source of light.

Milk samples were precipitated and filtered according to A.O.A.C. directions *(2)*. A 400-mm. tube was used for obtaining readings which were then converted into per cent lactose, using the following formula:

$$\text{Per cent lactose} = \frac{\text{observed angle of rotation} \times \text{dilution factor}}{\text{angle of specific rotation} \times \text{length of tube in decimeters}},$$

wherein the dilution factor was derived by dividing the total weight of sample and of reagents used by the weight of the sample.

RESULTS

The data (Table 1) show the reciprocity of ionic concentration to lactose in normal and mastitic milk. Although the mastitic milk contained only 3.75% lactose in contrast to 4.65% in the normal milk, the freezing points were −0.543 and −0.542° C., respectively, the higher ionic concentration compensating for the lower lactose in the mastitic milk.

Average values of seven trials with Jersey and Holstein College herd milk yielded freezing points of −0.540 and −0.542° C., respectively, as shown (data Table 2). The Jersey herd milk was lower in conductivity and higher in lactose than was the Holstein herd milk. Both herds were fed identical rations and were sampled on the same days.

A statistical analysis made on the 100 samples considered in this study indicates that the increase in accuracy in estimating freezing-point depression, attained by considering conductivity at 37° C. as well as lactose content, is not

TABLE 1

Influence of mastitis upon conductivity, per cent lactose, and freezing point of milk

Cow	Clinical condition of quarter	Conductivity at 37° C.	Lactose	Freezing point
(No.)		*[(mhos/cm)× 10^4]*	*(%)*	*(− ° C.)*
1	Normal	56.3	4.65	0.542
2	Inflamed (advanced)	59.5	4.30	0.541
3	Inflamed (acute)	68.6	3.75	0.543

TABLE 2

Comparison of conductivity, per cent lactose, and freezing point in Jersey and Holstein herd milk

	Conductivity at 37° C.		Lactose		Freezing point	
Trial	Jersey	Holstein	Jersey	Holstein	Jersey	Holstein
(No.)	*[(mhos/cm)× 10^4]*		*(%)*		*(− ° C.)*	
1	50.4	59.2	4.75	4.65	0.540	0.544
2	47.6	56.5	4.70	4.65	0.541	0.546
3	49.4	56.5	4.65	4.65	0.541	0.545
4	48.4	55.2	4.75	4.70	0.540	0.545
5	49.4	55.9	4.70	4.65	0.540	0.540
6	47.1	56.5	4.70	4.60	0.533	0.533
7	46.6	54.0	4.75	4.65	0.541	0.541
Av.	48.4	56.3	4.70	4.65	0.540	0.542

TABLE 3

Influence of decreasing per cent lactose in milk upon its conductivity and freezing point

Lactose in milk	Conductivity of the same milk at temperatures of		Difference in conductivity	Decrease in conductivity	Freezing point of milk
	37° C.	0° C.			
(%)	*[(mhos/cm)× 10^4]*		*[(mhos/cm)× 10^4]*	*(%)*	*(− ° C.)*
5.00	51.8	23.0	28.8	55.7	0.558
4.90	53.6	23.6	30.0	56.0	0.556
4.80	55.2	24.2	31.0	56.0	0.557
4.70	57.8	25.4	32.2	55.7	0.554
4.60	59.2	26.8	32.4	54.7	0.552
4.50	53.6	23.2	30.4	56.7	0.538
4.35	57.2	24.8	32.4	56.6	0.523
4.15	60.6	26.0	34.6	57.6	0.523
4.05	61.2	25.8	35.4	57.8	0.524
3.95	63.6	27.6	36.0	56.6	0.513
3.80	66.8	28.4	38.4	57.5	0.522
3.70	65.2	27.4	37.8	58.0	0.527

worth while, because the correlation between lactose content and freezing-point depression was 0.729, and including conductivity in a multiple correlation system yielded a multiple correlation of only 0.7292. The data (Table 3, Columns 1 and 6), obtained from a representative series of milk samples arranged in order of decreasing lactose content, demonstrates the influence of per cent lactose upon the freezing point of milk.

Of the 38 samples with 4.5% lactose or less, none had a freezing point of −0.550° C. or below, and only 16 samples met the 3% added water tolerance levels of −0.5335° C.; whereas, of the 62 samples containing above 4.5% lactose, 23 samples had freezing points of −0.550° C. or below, and only six samples fell below the 3% added water tolerance level.

Prolonged holding of raw milk at 0° C. up to 48 hr. resulted in slight decreases in conductivity and in a rise in the freezing points of herd milk (as shown

TABLE 4

Influence of prolonged holding of herd milk at 0° C. upon its conductivity and freezing point

Trial	Conductivity of milk after holding at °C. for: 1 hr.	48 hr.	Difference in	Freezing point of milk after holding at 0° C. for: 1 hr.	48 hr.	Difference
(No.)	*[(mhos/cm)× 10⁴]*		*[(mhos/cm)× 10⁴]*	*(− ° C.)*		*(°C.)*
			Jersey milk			
1	22.1	21.7	−0.4	0.541	0.539	−0.002
2	20.7	20.2	−0.5	0.540	0.537	−0.003
3	21.6	21.6	0.0	0.540	0.538	−0.002
4	22.0	21.7	−0.3	0.533	0.531	−0.002
5	21.7	21.7	0.0	0.541	0.539	−0.002
Av.	21.62	21.38	−0.24	0.539	0.5368	−0.0022
			Holstein milk			
1	25.4	25.2	−0.2	0.545	0.545	0.0
2	24.7	24.2	−0.5	0.545	0.541	−0.004
3	24.7	24.7	0.0	0.540	0.540	0.0
4	25.2	25.2	0.0	0.533	0.531	−0.002
5	24.5	24.2	−0.3	0.541	0.540	−0.001
Av.	24.9	24.7	−0.2	0.5408	0.5394	−0.0014

TABLE 5

Influence of pasteurization and overpasteurization upon the conductivity and freezing point of milk

Trial	Conductivity of milk at 25° C. when pasteurized for 16 sec. at: Raw	161° F.	169° F.	Difference between raw and pasteurized	Freezing point of milk when pasteurized for 16 sec. at: Raw	161° F.	169° F.	Difference between raw and pasteurized
(No.)	*[(mhos/cm)× 10⁴]*			*(169° F.–16 sec.)*		*(− ° C.)*		*(169° F.–16 sec.)*
1	45.8	45.0	44.8	− 1.0	0.533	0.528	0.526	−0.007
2	44.2	43.5	42.8	−1.4	0.535	0.528	0.524	−0.011
3	43.9	43.1	42.4	−1.5	0.535	0.527	0.525	−0.010
4	43.9	43.6	42.8	−1.1	0.534	0.529	0.527	−0.007
5	43.9	42.8	42.0	−1.9	0.535	0.531	0.529	−0.006
Av.	44.3	43.6	42.9	−1.4	0.534	0.529	0.526	−0.0082

in the data, Table 4). The changes observed were slightly greater in Jersey than in Holstein herd milk.

Data on the influence of pasteurization and overpasteurization of herd milk upon its conductivity and freezing point are shown (Table 5). Pasteurization at 161° F. for 16 sec. resulted in significant decreases in conductivity as well as in increases in freezing point. Overpasteurization at 169° F. for 16 sec. decreased the conductivity and increased the freezing point beyond the extent observed with properly pasteurized milk.

DISCUSSION

The interrelationship between conductivity and per cent lactose and its influence upon the freezing point of milk are evident from the data presented. Earlier findings *(1)*, that mastitic milk may have freezing points similar to those of normal milk, are substantiated in this study. But apparently this is not the case with low-lactose milk from nonmastitic animals. A possible explanation for this may be the difference in salt composition of the two types of milk. None of the low-lactose nonmastitic milk was salty to taste, whereas the mastitic milk was.

Both milk fat *(6)* and protein *(7)* have been reported to lower the conductivity of milk. This may explain, in part, the lower conductivity readings obtained with Jersey milk in contrast to those obtained with Holstein milk. Another factor known to affect electric conductivity in milk is its temperature. Temperature affects the extent of dissociation of molecules and also the rate of movement of ions *(5)*. Thus, Gerber *(6)* found the specific conductivity of milk to increase at the rate of one (mhos/cm) $\times 10^4$ per one degree rise in temperature between 10 and 30° C. The present study gave a calculated average increase in specific conductivity of 0.913 (mhos/cm) $\times 10^4$ with a range of from 0.78 to 1.075 (mhos/cm) $\times 10^4$ per degree C. Such values are in close agreement with Gerber's results, considering that the present study covered a temperature range of from 0 to 37° C.

Although a slight decrease in conductivity accompanied by an increase in the freezing point, after prolonged holding of raw milk at 0° C., may not be of practical significance, nevertheless it demonstrates that prolonged holding of milk may alter its freezing point. It is conceivable that fresh raw milk with a high initial freezing point, refrigerated for 48 hr. or more and overpasteurized, may have a freezing point above that permitted by law. The present trend in industry to prolong the life of milk, both in the raw state by low-temperature refrigeration, and by overpasteurization, may well lead to undesirable results from the standpoint of freezing point of milk. Although dilution of milk during processing may at times be responsible for a higher freezing point, this is not necessarily so.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. R. R. Shrode for the statistical analysis of data. Thanks are also due Dr. F. W. Jensen for his advice concerning conductivity measurements of milk.

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A COMPARISON OF DETERGENT TESTS FOR BUTTERFAT IN MILK WITH OFFICIAL METHODS

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SUMMARY

A collaborative comparison of the Schain and Dairy Products Section (DPS) tests with the official methods, the Babcock and Mojonnier tests, was performed by a group of experienced research workers. The volumetric tests were performed in duplicate by eight men; the Mojonnier test in quadruplicate by one man. Twelve cows, comprising four breeds with three cows of each breed, were used. Morning and evening milk was analyzed one day of the first, third, and fifth week.

The Babcock test has a closer correlation with the Mojonnier test than does either the Schain or the DPS test. The Schain test correlation is .9889, the DPS .9886, and the Babcock .9963, whereas a perfect correlation is 1.000.

The three volumetric tests averaged slightly higher than the Mojonnier: the Schain test 0.11% butterfat, the DPS 0.06%, and the Babcock 0.09%.

The standard deviation between testers was 0.15%, 0.18%, and 0.10% for the Schain, DPS, and Babcock tests, respectively. Therefore, the tester is a significant variable in these tests.

The butterfat content of the milk from these individual cows varied considerably, up and down, over the 5-wk. period. Differences of 1% fat were found in five out of the 12 cows, using the average of the morning and evening samples. This variation is far greater than that attributable to the analytical procedures or to the individual testers.

The dairy industry in the United States has relied on gravimetric analysis (Roese-Gottlieb or Mojonnier) and volumetric analysis (Babcock) for butterfat in milk for many years. Through continued research and collaborative study, these methods have been developed into highly accurate standard procedures. They are official methods of the Association of Official Agricultural Chemists and are incorporated in the laws of many states.

Alternative fat tests have been studied previously *(2, 8, 10, 11, 17, 18)*, but the development of tests that employ synthetic detergents was stimulated by the work of Schain *(15, 16)*, published in 1949 and 1950. Later work by Sager

Received for publication October 1, 1957.

[1] In collaboration with R. Bassette, M. Keeney, J. F. Mattick, and H A. Newlander (present address: Meadow Gold Products Co., Washington, D. C.), Dairy Husbandry Department, University of Maryland, College Park, Maryland; F. M. Grant and R. Trimble (retired), Animal Husbandry Research Division, Agricultural Research Service, USDA, Beltsville, Maryland; E. M. Parrish, Department of Public Health, District of Columbia, Washington, D. C., and H. E. Vettel, Eastern Utilization Research and Development Division, Agricultural Research Service, USDA, Washington, D. C.

and coworkers *(12, 13, 14)* resulted in the Dairy Products Section (DPS) detergent test. Both of these tests avoid the use of strong sulfuric acid, a definite liability of the Babcock test. The DPS test requires more equipment and is more complicated to perform than the Schain test, but avoids the use of the empirical nomogram required to calculate the results of the latter test.

A survey of the published results of these detergent tests *(3-7, 12-16)*[2] revealed a need for a careful comparison of them with the official methods. The experiment reported herein was, therefore, designed and carried out cooperatively by the EURDD, Animal Husbandry Research Branch, District of Columbia Health Department, and the Dairy Husbandry Department of the University of Maryland. The contribution of the University of Maryland must be especially acknowledged for, besides providing the analytical work of the four testers, the samples of milk, and the calibration of glassware, it performed other essential parts of the experiment. The design of the experiment was developed by the Biometrical Services, ARS, in cooperation with AMS. The Biometrical Services analyzed the data.

EXPERIMENTAL DESIGN

The experiment was designed to compare the accuracy of the Schain, the Dairy Products Section (DPS), and the Babcock tests with the Mojonnier (Roese-Gottlieb) ether extraction method for determination of butterfat; and to determine the factors affecting the precision of these methods in the hands of competent analysts.

In order that an accurate, statistical comparison of these methods would be made, it was decided to test 12 cows, comprising four breeds, with three cows of each breed. The three within each breed, i.e., three Guernsey, three Holstein, three Ayrshire, and three Jersey, were chosen as far as possible to be high, medium, and low fat producers of the respective breed. Morning and evening milk of each cow being tested was analyzed for butterfat content.

As required by the design, nine testers were assigned to perform the analyses. One tester analyzed every sample for butterfat content by means of the Mojonnier method. These determinations were performed in quadruplicate by Tester A. The results of these tests were considered to be the actual per cent of butterfat in the sample being tested. All results of the other methods of butterfat determination studied were compared with these Mojonnier results.

The eight other testers, B, C, D, E, F, G, H, and I, analyzed every sample by the methods of Schain, Dairy Products Section, and Babcock butterfat tests, except for one set not analyzed because of illness and the unavoidable loss of a few samples. The tests were run in duplicate; the results were recorded as individual readings for each test performed, rather than as averages of the duplicates.

The experiment was conducted over a 6-wk. period consisting of 3 wk. of

[2] The valuable study of E. O. Herreid and collaborators (J. Assoc. Offic. Agricultural Chemists, 40: 499. 1957.) was reported after the completion of the experimental work reported herein. The results of the two studies are in general agreement but, because of their differing purposes, can not be closely correlated.

testing and 3 wk. of assembling the data. The 1st, 3rd, and 5th wk. were the actual testing weeks. The experimental program for testing was as follows: On the first day of the first testing week (Tuesday, October 3, 1955), the Guernsey breed was tested, i. e., the morning *(m)* and evening *(e)* milks of the high, medium, and low test cows of the Guernsey breed were analyzed by Tester A, who used the Mojonnier method of testing (see Methods of Analysis). The other eight testers, B, C, D, E, F, G, H, and I, tested aliquots by the Schain, DPS, and Babcock tests. On the second day of the first testing week, the same procedure was carried out with the Holstein breed; on the third day with the Ayrshire breed, and one the fourth day of testing the Jersey breed was tested in a similar manner. In the 2nd and 3rd wk. of testing the same plan was followed.

PROCEDURE FOLLOWED IN CARRYING OUT THE STUDY

A. *Equipment and materials.* The equipment was carefully selected and checked, since emphasis was placed on accuracy. The glassware was standardized by the Dairy Inspection Service of the State of Maryland. All glassware which did not meet the requirements of the State was discarded. It was also ascertained that the speed of weighing might also be a factor in the accuracy of the tests to be performed. Therefore, Mojonnier dishes were purchased which were tared to within 3 mg. of each other. Thus, only a limited amount of weight-adjusting was necessary for each aliquot of each sample, which was accomplished on a rapid indicating balance.

The chemicals and reagents used in the testing study were as follows: The sulfuric acid used in making the fat determinations by the Babcock method had a specific gravity of 1.84; therefore, each tester had to standardize his own acid to a specific gravity of 1.82-1.83, which is the specific gravity of the sulfuric acid designated Babcock acid. The Schain reagent was obtained in one lot of eight individual gallons from Dr. Philip Schain of Staten Island, N. Y. The sodium tetraphosphate (Quadrafos)[3] was obtained from the Rumford Chemical Company, Rumford, Rhode Island, and the methyl alcohol (99.6%) from Mojonnier Brothers,[3] Chicago, Illinois. The alcohol was standardized by the individual testers to 50%. Triton X-100[3] and Quadrafos were obtained in a large container and portions were distributed to the testers, because these chemicals were not available in small lots. All other chemicals were purchased in individual containers. Each tester was supplied with the necessary equipment (except the centrifuge and water baths) and chemicals to carry out the tests. This was done for the purpose of keeping conditions as similar as possible, to minimize sources of error.

B. *Methods of analysis.* A working handbook was prepared for each tester, that contained the method and step-by-step procedure for each test. The directions for the Babcock test were taken directly from official methods *(1)*. The Mojonnier test was run according to directions published by the company *(9)*,

[3] The mention of commercial products and manufacturers is for the purpose of identification and does not constitute endorsement by the USDA.

except that three extractions were performed and the final extraction of the fat was made using only petroleum ether and ethyl ether. This modification made the procedure essentially equivalent to the Roese-Gottlieb method, as described on page 233 of the AOAC methods *(1)*.

Testers were instructed to read their results as accurately as possible to the second decimal place, for the Babcock and for the two other volumetric tests. Directions for the DPS test were taken from the paper of Sager *et al. (14)*. The Schain test was performed according to directions described in photostatic copies of the method, as revised February 1955, with minor inked-in changes made by Dr. Schain just prior to the initiation of this study.

C. *Manipulation of samples.* The evening milk samples were obtained from the University of Maryland dairy barns by one of the cooperating testers. The milk was obtained by machine-milking and placed in individual milk cans. It was immediately taken to the dairy manufacturing building, agitated thoroughly by pouring from one container to another three times, then 9-pt. bottles of each sample were poured. The pint aliquots were stored overnight at 40° F.

The morning milk was handled in an identical fashion, except that it was not stored but was delivered to the cooperating testers as soon as possible, usually by 8 A.M. Both sets of samples were warmed to from 105 to 110° F., mixed thoroughly in the bottles, and then tempered to the standard temperatures of analysis before pipetting the samples.

RESULTS

The data before analysis are not presented, because of their great number. Photostats of the tabulated results can be obtained by writing to the senior author.

Averages of the volumetric analyses by the eight testers, for each sample plotted against the Mojonnier data, are shown (Figure 1). Certain features of the data, such as the fact that most of the samples had fat contents between 3 and 6%, and that the standard deviation for the DPS and Schain tests is greater than for the Babcock test, are apparent by inspection of this figure.

The experimental design called for the collection and analysis of 72 samples of milk; two from each of the 12 cows in each of the three testing weeks. However, three samples were not analyzed, because they were either lost or churned before reaching the tester. In addition, some of the testers were unable to obtain determinations on several of the remaining 69 samples. The loss of determinations, as reported by the eight testers (B through I), with the Schain and DPS tests, was almost five times and three times as great, respectively, as with the Babcock test. Loss of determinations with the Babcock test was negligible.

The four determinations with the Mojonnier (by Tester A) on each of the 69 samples were averaged and compared with the averages of the duplicates for each of the other tests, separately, for each of the eight testers. In addition, the 69 samples were divided into a high and low group, based on the fat content as determined by the Mojonnier method. The high group consisted of 35 samples, with the average Mojonnier reading of the lowest sample being 4.234% fat and

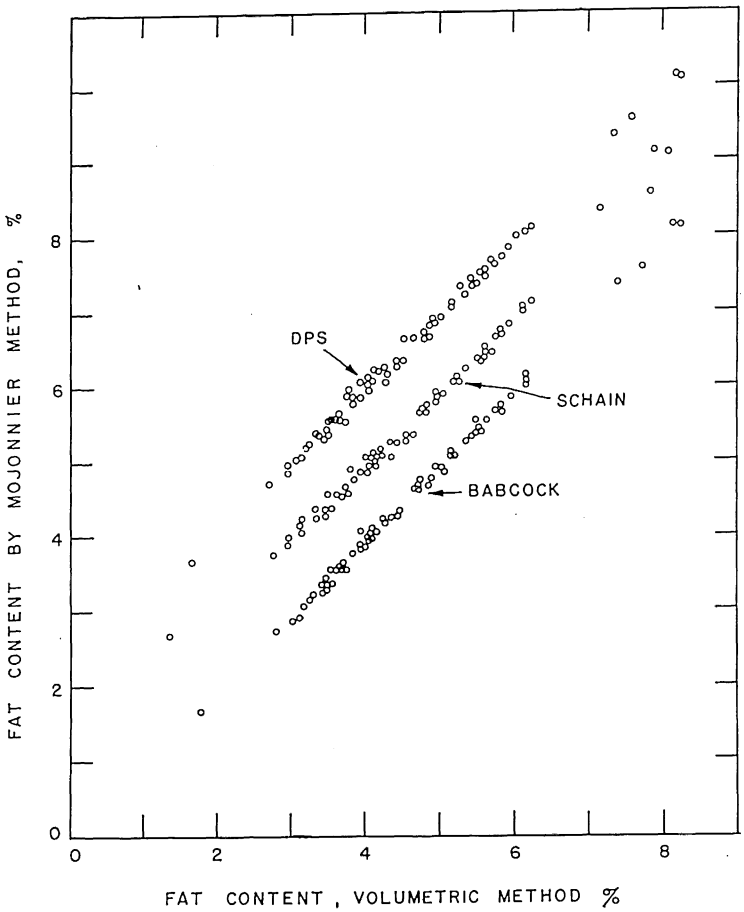


FIG. 1. Average of the volumetric analyses for the eight testers *versus* average of Mojonnier run in quadruplicate by one tester. The ordinate is raised by 1% for the Schain test and by 2% for the DPS test, that is, all points are arbitrarily raised by these amounts to separate the data for the three sets of results.

of the highest sample, 8.118% fat. The low group consisted of the 34 other samples, with the low sample containing 1.658% fat and the high sample, 4.218% fat. The reason for placing the samples into two groups was to determine if the linear regression of the Mojonnier reading on the Schain, DPS, or Babcock tests differed at different fat levels, which would be good evidence of the existence of curvilinearity. A summary of the linear regression and simple product-moment correlation coefficients is given (Table 1).

The difference between the regression in the low group and regression in the high group is 0.175, 0.046, and 0.022 for the Schain, DPS, and Babcock tests, respectively. These results suggest that the true relation between the Mojonnier and Schain tests can best be described with a curved, rather than a straight, line. The relation between the Mojonnier and DPS tests appears to be only slightly curvilinear; whereas, the Mojonnier and Babcock tests are essentially linear in

TABLE 1

Intratester correlations and regressions of the Mojonnier Test with the Schain, DPS, and Babcock Tests

Groups based on fat content	Schain	DPS	Babcock
		Regressions	
Low	0.851	0.916	1.011
High	1.026	0.962	0.989
All samples	0.964	0.961	0.991
		Correlations	
Low	0.9647	0.9533	0.9815
High	0.9798	0.9774	0.9936
All samples	0.9889	0.9886	0.9963

their relation to each other. Additional samples are needed, especially in the upper and lower fat levels, in order to establish these relations accurately.

It should also be noted (Table 1) that the Babcock test is more closely correlated with the Mojonnier reading than with the two other tests. Although these differences are not great, they are responsible for differences in the standard errors of estimate, which are significant statistically (as can be seen in Table 2). The linear regression and simple product-moment correlation coefficients of the Mojonnier test with the Schain, DPS, and Babcock tests were calculated separately for each of the eight testers. These did not vary materially from one tester to another. However, the average difference of the Mojonnier test and each of the other tests varied widely between testers. These differences and the standard errors of estimate are given (Table 2) for all samples and for each tester separately. The standard deviation between testers is given at the bottom of the table for each test.

Only one tester (C) had a larger standard error of estimate for the Babcock test than for either of the two detergent tests. The standard errors of estimate for all 69 samples on an intratester basis were 0.17, 0.18, and 0.10 for the Schain, DPS, and Babcock tests, respectively.

The variability between testers within samples, as measured by the standard deviations (bottom of Table 2), originates from three possible sources. These are *(1)* differences that would exist between determinations (using the same test) on the same sample by the same tester, *(2)* real differences between testers, and *(3)* the interaction of samples and testers. The importance of these sources of variation in the bias observed between the Mojonnier test and each of the three other tests is more critically evaluated in subsequent analyses.

Duplicate determinations. The general procedure followed by most testers in the conduct of these tests was to repeat the determinations, when the first set of duplicate determinations failed to agree within the limit set in the instructions (0.10% fat).[4] The discarded determinations were not reported by the

[4] Although the directions for the Babcock test and the Schain test call for repetition of the test, where duplicates vary by more than 0.10% fat, and where those for the DPS test do also by inference, inspection of the raw data shows that this was not always done. The analysis of variability between duplicates is affected by these facts, but the validity of the major correlations is not affected significantly thereby.

TABLE 2

Average differences from the Mojonnier Test, standard errors of estimate, and standard deviations between testers for the Schain, DPS, and Babcock Tests

		Test		
		Schain	DPS	Babcock
	D[a]	0.14	0.18	0.20
Tester B	SE of E[b]	0.12	0.09	0.07
	D	0.13	0.10	0.06
Tester C	SE of E	0.15	0.14	0.16
	D	0.08	0.14	0.16
Tester D	SE of E	0.13	0.10	0.07
	D	0.12	0.04	0.08
Tester E	SE of E	0.19	0.27	0.11
	D	0.08	−0.01	0.13
Tester F	SE of E	0.15	0.23	0.12
	D	0.07	−0.03	0.11
Tester G	SE of E	0.20	0.17	0.06
	D	0.15	0.09	0.03
Tester H	SE of E	0.13	0.19	0.08
	D	0.12	−0.05	−0.07
Tester I	SE of E	0.21	0.14	0.12
	D	0.11	0.06	0.09
All:	SE of E	0.17	0.18	0.10
	SD[c]	0.15	0.19	0.12

[a, b, c] D refers to differences from the Mojonnier, SE of E to standard errors of estimate, and SD to standard deviations between testers.

tester. The standard deviation between duplicate determinations was calculated for each tester and for the Schain, DPS, and Babcock tests separately. These standard deviations are shown (Table 3), along with the number of samples that were reported with duplicate determinations in each case.

The variability between duplicate determinations varied between testers. On the average, the greatest difference occurred between duplicate determinations that were reported for the DPS test (standard deviation =0.073), intermediate for the Schain test (standard deviation =0.052), and smallest for the Babcock test (standard deviation =0.046).

Variability among daily means. Because of missing values in some cases, it was necessary to average over the morning and evening samples from the same cow to simplify the analyses of variance. Data collected by the last tester (I) were omitted from these analyses, because he made no determinations on 12

TABLE 3

Standard deviations between duplicate determinations and number of samples in each case

	Standard deviations			No. samples		
	Test			Test		
	Schain	DPS	Babcock	Schain	DPS	Babcock
Tester B	0.035	0.044	0.027	67	69	69
Tester C	0.035	0.053	0.042	67	68	68
Tester D	0.050	0.040	0.025	65	68	68
Tester E	0.062	0.134	0.032	67	68	68
Tester F	0.044	0.041	0.090	57	57	68
Tester G	0.033	0.036	0.034	61	67	68
Tester H	0.077	0.106	0.024	67	68	68
Tester I	0.062	0.060	0.055	58	58	58
Intratester	0.052	0.073	0.046	509	523	535

samples. Both a morning and an evening sample were collected and tested for each cow, except in four instances, where one of these two samples either was not collected or had no readings by most testers. The table of daily means, set up from the original data, was a tester × test × cow × week table. The corresponding daily averages of the Mojonnier test were then subtracted from each of these values. This gave a total of 756 mean differences (biases) from the Mojonnier. These biases were analyzed by the analysis of variance for each breed and for each test separately. The mean squares of the analyses of variance for each test are given (Table 4) and the separate analyses of variance from each of the four breeds are shown (Table 5).

It should be noted (Table 4) that the error variance (M × W × C : B) is three times and five times larger, for the Schain test and DPS test, respectively,

TABLE 4

Analysis of variance of biases for each test, mean squares only[a]

	d.f.	Test		
		Schain	DPS	Babcock
Total	251	0.0368	0.0393	0.0172
Breeds	3	0.0452	0.1760	0.0174
Men (M)	6	0.0376	0.2019	0.1352[b]
Weeks (W)	2	0.1621	0.0356	0.0540
B × M	18	0.0204	0.0392	0.0074
B × W	6	0.1309	0.0232	0.0621
M × W	12	0.0559[b]	0.0798[b]	0.0246[b]
B × M × W	36	0.0246[b]	0.0450[b]	0.0078[b]
Cows (C:B)	8	0.2840	0.1303	0.1042
M × C:B	48	0.0085	0.0147	0.0024
W × C:B	16	0.1382[b]	0.0851[b]	0.0630[b]
M × W × C:B	96	0.0098	0.0160	0.0031

[a] Appropriate error terms were determined by assuming that all effects except breeds were random.

[b] Significant at the 0.01 level.

TABLE 5

Analyses of variance of biases for each breed, mean squares only[a]

	d.f.	Breed			
		Guernseys	Holsteins	Ayrshires	Jerseys
Total	188	0.0646	0.0323	0.0118	0.0147
Men (M)	6	0.0604	0.0467	0.0396	0.0809
Tests (T)	2	0.0681	0.1367	0.0349	0.0050
Weeks (W)	2	0.5938	0.0082	0.0505	0.0386
Cows (C)	2	1.5131	0.1107	0.0682	0.0281
M × T	12	0.0774[b]	0.0631	0.0077	0.0260
M × W	12	0.0605[c]	0.0363[b]	0.0171	0.0308[c]
M × C	12	0.0137	0.0097	0.0040	0.0048
T × W	4	0.0474	0.0076	0.0211	0.0284
T × C	4	0.0579	0.0880[b]	0.0077	0.0232
W × C	4	0.9208[c]	0.0343[b]	0.0848[c]	0.0139
M × T × W	24	0.0292[c]	0.0493[b]	0.0142[c]	0.0301[c]
M × T × C	24	0.0070	0.0218	0.0040	0.0025
M × W × C	24	0.0093	0.0122	0.0080[b]	0.0053[b]
T × W × C	8	0.0190[c]	0.0144	0.0048	0.0077[b]
M × T × W × C	48	0.0054	0.0279	0.0043	0.0028

[a] Appropriate error terms were determined by assuming that all effects except tests were random.

[b] Significant at the 0.05 level.

[c] Significant at the 0.01 level.

than is the error variance for the Babcock test. These differences in error variances are significant statistically, and clearly show that sources of variation other than those considered in this study were considerably more important in the Schain and DPS test than in the Babcock test. Perhaps this can be explained by the fact that the conditions and manipulations of the Babcock test have been more highly standardized, through long study and use, than have those of the newer tests.

The week × cow interaction within breeds (W × C:B) is highly significant for all three tests (Table 4). This means that the bias in estimating the average fat content of two samples (in most cases—morning and evening) on the same cow varied from week to week, even when the same test was used. The week × cow interaction, when averaged over all tests, but separately for each breed (Table 5), is significant for the Holstein data and highly significant for the Guernsey and Ayrshire data. Changes in the average bias from week to week for data from the same cow are shown (Table 6). This interaction is more noticeable in the Guernsey breed because of one low sample collected on Cow No. 1 the first week, which was greatly underestimated on the average by the Schain test.

Analyses of variance similar to those given in Tables 4 and 5 were calculated for the daily means without regard to the Mojonnier readings. The week × cow interaction was highly significant in all of these analyses. Therefore, because the fat content of samples from these cows varied differentially by weeks and because an association was found between the amount of bias and the fat content

TABLE 6

Subclass means of biases

Breed	Cow	Weeks 1	2	3	Test Schain	DPS	Babcock	Means
	1	−0.500	0.061	0.104	−0.141	−0.117	−0.077	−0.112
	2	0.209	0.186	0.138	0.267	0.114	0.152	0.178
	3	0.151	0.140	0.096	0.144	0.086	0.157	0.129
Guernsey		−0.047	0.129	0.113	0.090	0.028	0.077	0.065
	1	0.017	0.087	−0.010	−0.015	−0.009	0.118	0.131
	2	0.029	0.033	0.024	0.114	−0.074	0.047	0.029
	3	0.087	0.080	0.141	0.102	0.091	0.115	0.103
Holstein		0.045	0.067	0.052	0.067	0.003	0.093	0.054
	1	0.095	−0.005	0.061	0.118	0.051	0.082	0.084
	2	0.118	0.088	0.061	0.098	0.071	0.099	0.089
	3	0.190	0.151	0.088	0.178	0.135	0.116	0.143
Ayrshire		0.134	0.078	0.104	0.131	0.085	0.099	0.105
	1	0.065	0.155	0.115	0.097	0.123	0.115	0.112
	2	0.120	0.144	0.140	0.165	0.117	0.122	0.135
	3	0.094	0.123	0.060	0.046	0.115	0.116	0.092
Jersey		0.093	0.141	0.105	0.103	0.118	0.118	0.113
Means		0.056	0.104	0.093	0.098	0.059	0.097	0.084

	Tester B	C	D	E	F	G	H
Schain	0.130	0.108	0.062	0.107	0.065	0.070	0.141
DPS	0.164	0.085	0.114	0.018	−0.017	−0.044	0.091
Babcock	0.191	0.047	0.150	0.065	0.114	0.097	0.013
Week 1	0.098	0.010	0.090	0.052	0.045	0.047	0.052
Week 2	0.193	0.098	0.098	0.100	0.025	0.088	0.124
Week 3	0.194	0.133	0.139	0.038	0.092	−0.112	0.069
Means	0.162	0.080	0.109	0.063	0.054	0.041	0.082

of the sample in these data, it seems safe to conclude that a considerable amount of the week × cow interactions would be eliminated, if adjustment were made for the level of fat.

The breed × men × week (B × M × W) interaction is highly significant for all three tests (Table 4). This means that the average weekly changes in the biases from one breed to another were not evaluated in the same manner by all testers. The Babcock test was considerably less variable from tester to tester, in this respect, than were the two other tests.

The highly significant men × week (M × W) interaction for each of the three tests (Table 4) indicates that these tests were not conducted in the same manner by each tester each week. Changes that took place in the test procedures, reagents, etc., were not the same for all testers. The men × week (M × W) interaction, when averaged over all tests for each breed separately (Table 5), is significant for the Holstein data and highly significant for the Guernsey and Jersey data.

Experience in collaborative studies of this type has shown repeatedly that analytical tests are more sensitive to subjective effects than it is commonly believed. The significance of the M × W interaction indicates clearly that the individual operator is from day to day a variable in the performance of these tests, regardless of extensive efforts to standardize procedures, reagents, equipment, etc., between testers.

The greatest differences between testers occurred with the DPS test. However, these differences varied so much from week to week with the DPS test that the average differences between testers are not quite significant at the 0.05 level, when the effects of weeks are considered as random. On the other hand, the average differences between testers are highly significant in the case of the Babcock test, because of less variation in these differences from week to week.

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EARLY EMBRYOLOGY OF THE COW.
I. GASTRULA AND PRIMITIVE STREAK STAGES[1, 2]

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SUMMARY

Serial sections of 16 bovine embryos recovered from cows slaughtered at 16, 17, and 18 days after insemination were studied to establish suitable standards of normal development and to define the limits of normal variation encountered during early pregnancy. The 16-day-old blastocysts were engaged in the processes of gastrulation, and ranged in development from a hollow bilaminar sphere to an elongate chorionic vesicle with an elevated germinal disk. Their principal features were the remarkably rapid growth of the trophoblast and the differentiation of somatic and splanchnic mesoderm. Blastocysts recovered at 17 days were characterized by further growth, cephalocaudal elongation of the germinal disk, and differentiations of Hensen's node and the primitive streak establishing the longitudinal axis. In the 18th day of development, the blastocysts extended throughout the major portion of the gravid horn and the embryos displayed well-defined primitive node, primitive groove, and notochord development. Amniogenesis was initiated and, in two specimens, completed amniotic sacs were present. Two cases of probable embryonic death were encountered in which the trophoblast persisted after loss and resorption of the embryonic area. The morphogenetic processes involved in gastrulation and primitive streak formation are considered in the light of reported high embryonic death during early pregnancy.

The literature pertaining to studies of the embryology of the cow is notably sparse. Hallman *(11)* studied the development of placentae from cows from 3 to 5 mo. pregnant, with special reference to placental pathology. Hammond *(13)* studied the relations between rate of development of the uterus, fetus, fetal fluids, and fetal membranes, and included brief descriptions and illustrations of a few fetal specimens recovered at the end of each lunar month of pregnancy. Hartman *et al. (14)* and Miller *et al. (23)* described a two-celled bovine egg recovered 48 hr. after mating and an unfertilized tubal ovum 72 hr. after mating. Yapp *(31)* presented data on 31 fetuses ranging from 41 to 277 days of pregnancy, emphasizing changes in body proportions during various periods of development. Kupfer *(21)* reported on a series of specimens with estimated ages from about 3 to between 15 and 17 wk. of age.

The most valuable and precise information on the embryology of the cow is provided by Winters *et al. (30)*. Employing timed specimens, they presented data and illustrations on material ranging from one-day-old ova to the new-born calf. The scope of this undertaking made it necessary to base some observations

Received for publication June 3, 1957.

[1] Contribution No. 1107 of the University of Massachusetts College of Agriculture Experiment Station, Amherst, Massachusetts.

[2] Supported by the Northeast Cooperative regional project NE-I, "Causes and Prevention of Reproductive Failures in Dairy Cattle," and by funds from Animal Husbandry Research Branch, USDA, Beltsville, Maryland.

upon single specimens; moreover, there are a number of omissions in the day-by-day descriptions of the embryological development.

Nichols *(25)* collected data on body weights and a number of body measurements from about 100 embryos and fetuses of Hereford cows, but precise knowledge of the stages of gestation was lacking. A study of the rate of development of the gravid uterus and its contents at various stages of pregnancy was made by Swett *et al. (27)*, using data from 115 cows ranging from 14 to 276 days of gestation.

More recently, the development of the bovine ovum up to the stage of blastocyst formation has been reported by Hamilton and Laing *(12)*. Chang *(5)* described blastocysts obtained from slaughterhouse material estimated to range from about 8 to 18 days of development. Greenstein and Foley *(9, 10)* reported briefly on the development of the bovine embryo between the late blastocyst stage and early organogenesis.

The recognition of early embryonic death as an important factor in bovine infertility *(1, 3, 4, 6, 15, 16, 28, 29)* has pointed up the need for more detailed, accurate information relative to normal embryonic development during the critical stages of germ layer formation, the development of body form, and the establishment of the organ systems. This paper is the first in a series describing the day-by-day development of the cow from the late blastocyst stage to the fully formed embryo.

MATERIAL AND METHODS

Reproductive tracts were recovered from dairy and beef cows and heifers slaughtered at the University abattoir. These cows represented culls from the University herds. Detailed records were available relative to ancestry, health, age, breeding history, and reproductive performance of all individuals. The cattle were closely watched for signs of estrus, and those that came into estrus in the morning were mated at that time and again in the afternoon; those coming into heat in the afternoon were bred that day, and again the following morning if still in estrus.

Since ovulation normally occurs from 10 to 15½ hr. postestrum *(2, 13, 24)*, in calculating the ages of the embryos in this study the day after the last breeding was considered as the first day of pregnancy and the date of slaughter as the final day.

Specimens were flushed from the uterine horns with physiological saline for observation under the dissecting microscope. They were then photographed, or fixed immediately in a modified Bouin's fluid, followed by standard dehydration, clearing, and Tissuemat infiltration. The embryos were serially sectioned at 8 or 10 microns and stained with hematoxylin-eosin or a modified Mallory triple stain.

Observations. Sixteen timed embryos were available for study. A list of the embryos and their principal characteristics is presented (Table 1).

Sixteen-day embryos. Six embryos were recovered from animals slaughtered 16 days after breeding. The gross appearance of the specimens varied consider-

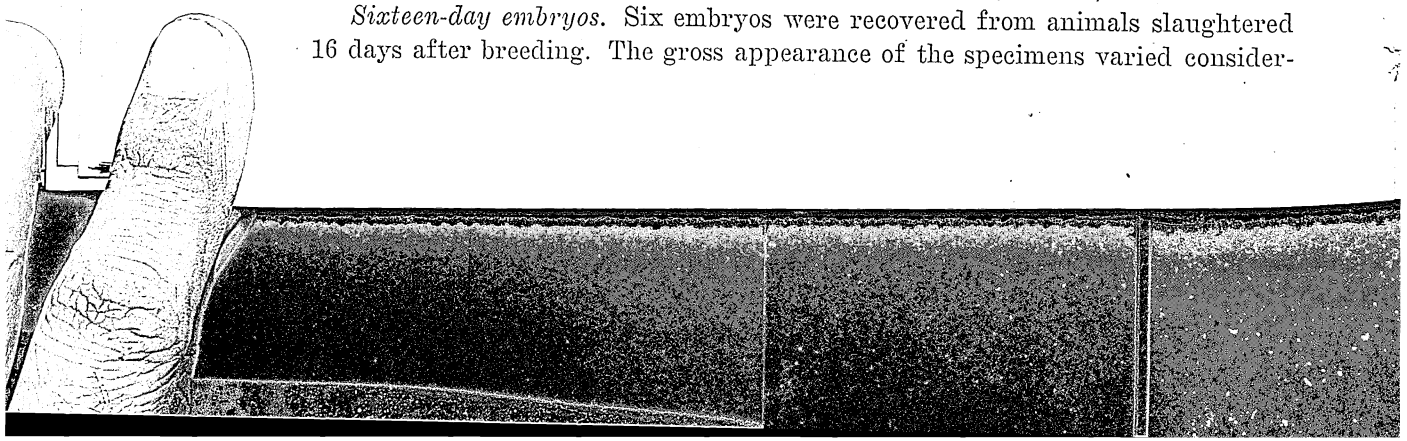


TABLE 1

Pregnant	Animal	Blastodermic vesicle	Stage of embryonic development	Figure
(days)	*(No.)*	*(mm.)*		
	NE-20	1.5	Bilaminar hollow sphere. Embryonic disk area distinct from trophoblast.	
	NE-93[a]	6	Early elongation of blastocyst. Dense spherical disk (0.4 mm.) elevated to surface.	1, 4, 5, 6, 7
	NE-69[b]	29	Marked growth and folding of trophoblast	
16			wall. Embryonic disk unchanged.	2
	NE-16	60	Slender vesicle (1.65 mm. in diameter). Disk occupies middle portion.	
	NE-132	75	Cephalocaudal elongation of disk (0.45 by 0.6 mm.). Proliferation of mesoderm.	3, 8, 9, 10, 11
	NE-22	90	Embryonic failure. Trophoblast undergoing degeneration.	
	NE-50	70	Embryonic failure. Persistent chorionic development.	
	NE-61[a]	125	Primary germ layers well-established. Ex-	
17			tension of extraembryonic mesoderm.	
	NE-148	135	Oval disk (0.4 by 0.75 mm.) with caudal primitive groove. Possible early amniogenesis.	12, 13, 14, 15
	NE-39	150	Longitudinal axis clearly evident. Undercutting of embryonic area.	16, 17
	NE-55	240	Pronounced Hensen's node and mesodermal delamination.	18, 19
	NE-49[a]	100	Thickened ectoderm, well-formed primitive streak. Disk 0.56 by 0.72 mm.	20
	NE-85	175	Distinct extraembryonic coelom. Sug-	
18			gestion of primitive amniotic folds.	21, 22, 23
	NE-90	Length of gravid horn	Marked primitive groove. Extensive extraembryonic coelom.	24
	NE-37	225	Well-defined amniotic sac. Emergence of notochordal elements.	25
	NE-131	Invading non-gravid horn	Well-developed amnion, notochordal mass, and lateral mesoderm.	26, 27

[a] Five or more services required for conception.
[b] Previous abortion.

ably, particularly with regard to the elongation of the chorionic vesicles, ranging from 1.5 to 90 mm. in length, with a mean length of 43.6 mm. The diameters of the vesicles were relatively constant at approximately 1.5 mm. The embryonic disks were similar in all specimens, being nearly spherical, with an approximate diameter of 0.4 mm.

Specimen NE-20 appeared as a minute, bilaminar, hollow sphere with only faint evidence of an embryonic disk on its surface. Unfortunately, this specimen was lost in processing. A somewhat larger blastocyst was recovered from NE-93, as illustrated (Figure 1). The embryonic disk was imperceptible in saline solution but, with the addition of fixative, became evident as an opaque spherical structure.

Figures 4 to 7 illustrate representative 8-micron sections of specimen NE-93. The germinal disk has become completely elevated to the surface, with only an occasional vestige of overlying trophoblast or Rauber's membrane (Figure 6) remaining as detached cells. The endoderm has separated from the germinal

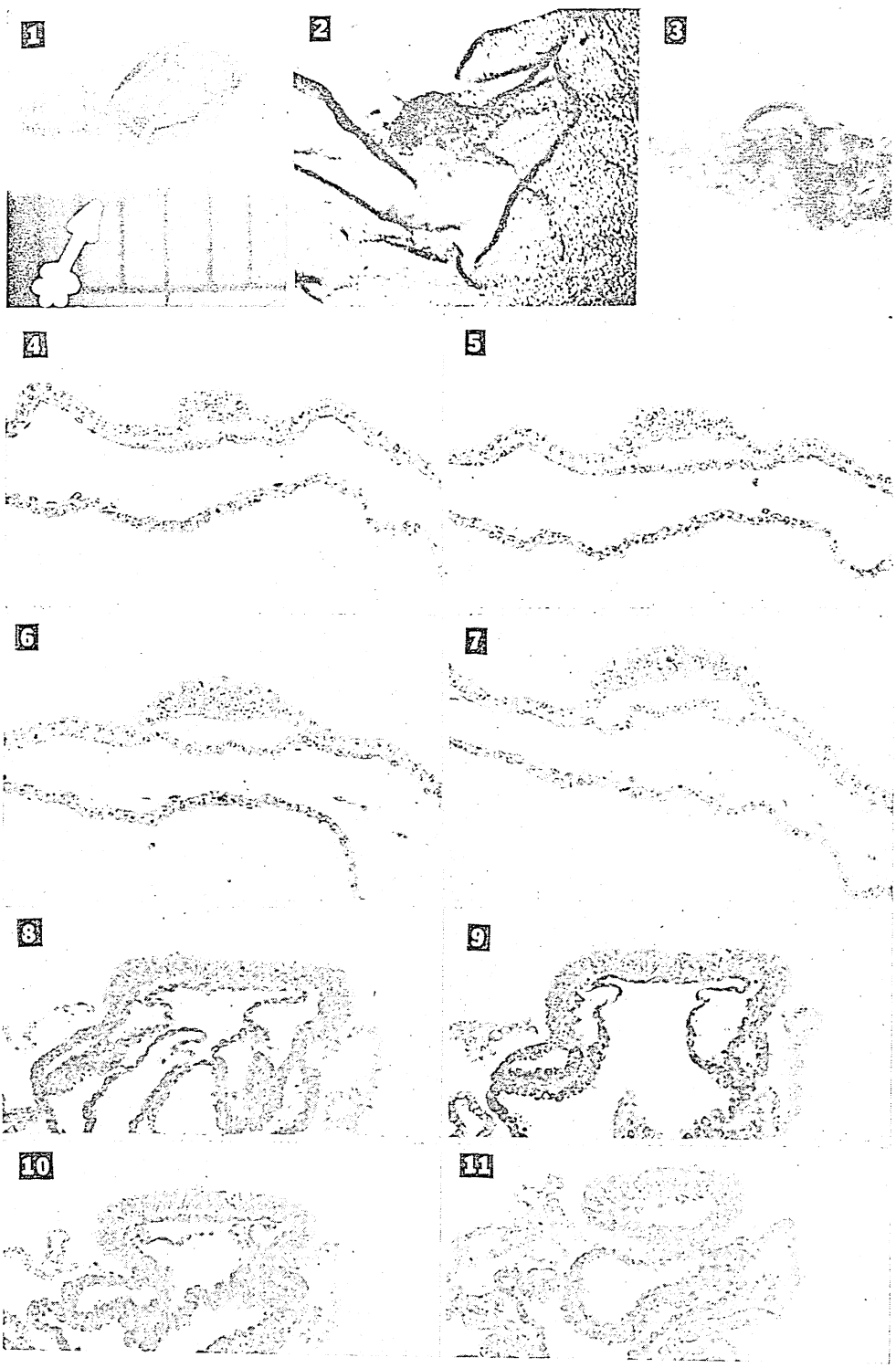


Plate 1

disk area and forms a complete lining for the blastocyst. Mitotic figures are seen frequently in the germinal disk and trophoblast, indicating a period of rapid growth and elongation.

Specimen NE-69 is shown in Figure 2. Although the chorionic vesicle was considerably longer than in the previous specimen, sections through the germinal disk indicated that the embryonic area did not differ significantly from NE-93. There was, however, a marked growth and folding of the wall of the trophoblast. The disk remained essentially spherical with a diameter of 0.35 mm. and was located in the mid-portion of the vesicle. There was no evidence of overlying trophoblast cells.

In Specimen NE-16 the trophoblast had elongated considerably, and the width of the chorion had increased to approximately 1.65 mm.

Figure 3 illustrates the prominent, raised, oval germinal disk of Specimen NE-132. The embryonic area was located approximately midway along the slender (1.5-mm. diameter) vesicle. Figures 8 to 11 are representative 10-micron sections taken at various levels through the germinal disk. This specimen shows a striking advancement over the specimen in Figures 4 to 7. The ectodermal disk is well-formed, and the mesoderm has separated to form the extraembryonic somatopleure, splanchnopleure, and coelom. Undercutting of the embryonic area indicates the formation of the future body stalk.

Although NE-22, when recovered, appeared to be the most advanced specimen of this 16-day-old group by virtue of its length, the embryonic disk was not discovered either upon gross examination or in tissue sections. Large numbers of pyknotic nuclei indicated that the trophoblast was undergoing degeneration, and it is reasonable to assume that the embryonic tissue proper had disappeared sometime prior to slaughter.

Seventeen-day embryos. Five embryos recovered 17 days after breeding were available for detailed study. The variation in appearance among the specimens was not as pronounced as it was on the previous day. The length of the chorion ranged from 70 to 240 mm., with a mean length of 144 mm. In all cases, the vesicle was somewhat enlarged in the central region, tapering toward the extremities. The embryo occupied a position in the approximate mid-portion of the vesicle, and at this stage there was a consistent tendency toward elongation of the germinal disk to assume an oval rather than a circular shape.

PLATE 1

1. Entire 16-day-old blastocyst NE-93 and millimeter rule photographed in saline. Arrow points to opaque embryonic disk. 6.2×.

2 and 3. Portions of elongate 16-day-old blastocysts, NE-69 and NE-132, showing characteristic raised germ disks on surface of trophoblast. 33× and 30×.

4, 5, 6, and 7. Transverse 8-micron sections of NE-93 (Figure 1) showing separation of endoderm layer from germ disk. Notice frequency of mitotic figures. Vestige of overlying trophoblast may be seen in Figure 6. 125×.

8, 9, 10, and 11. Transverse 10-micron sections of NE-132 (Figure 3). Note undercutting of embryonic area, the presence of a definitive yolk sac, and the delamination of mesoderm to form splanchnopleure, somatopleure, and extraembryonic coelom. 125×.

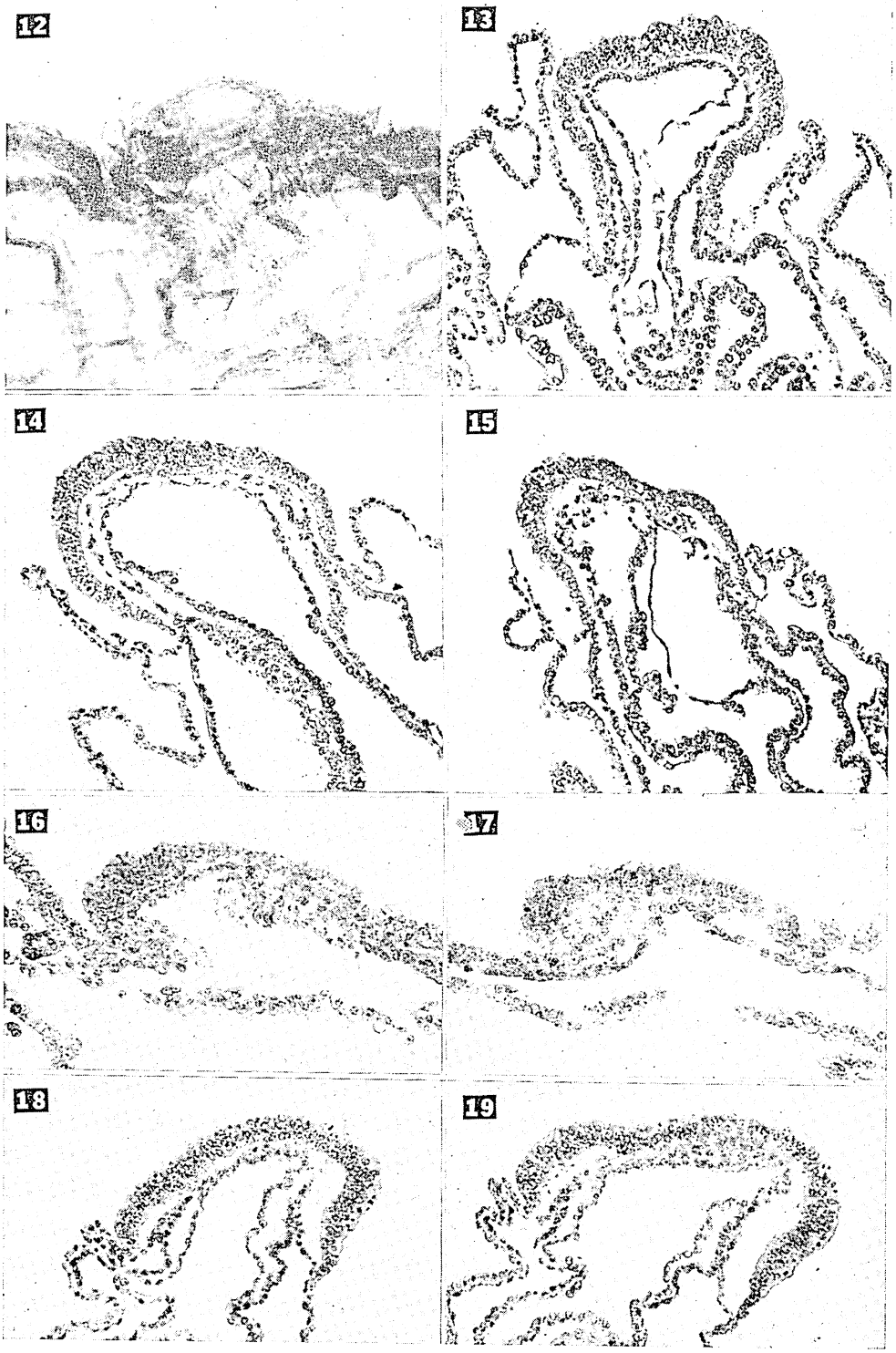


Plate 2

The embryonic area of the chorionic vesicle in specimen NE-50 was not positively identified either grossly or after serial sectioning. On the basis of microscopic examination of the tissue sections, it may be concluded that this specimen represents a case of persistent chorionic development in spite of prior embryonic failure. This conclusion is supported also by the relatively retarded length of the vesicle.

Specimen NE-61, whose germinal disk measured 0.6 by 0.8 mm., was located in the mid-portion of the vesicle. Sectioned at 8 microns, 98 sections were obtained. Aside from an over-all increase in the size of the disk, the appearance of the primary germ layers is essentially the same as in Specimen NE-132.

Figure 12 illustrates the gross appearance of Specimen NE-148. Sixty-eight sections were obtained at 10 microns and these revealed a well-developed mesoderm between the ectodermal plate and the yolk-sac endoderm. The lining of the cavity of the yolk-sac is composed of small, round cells with only a narrow rim of cytoplasm surrounding the oval, vesicular nuclei (Figures 13 and 14). The convex dorsal surface of the disk is rounded and smooth, except for evidence of a caudal primitive groove (Figure 15). The area of proliferation marking Hensen's node also may be seen in this photograph. The ectodermal cells with numerous mitotic figures rest on a basement membrane with the tall columnar cells gradually becoming cuboidal at the lateral borders of the disk. An abrupt transition to flat ectodermal cells is seen in the lateral body folds. There are indications that the embryonic disk has settled somewhat into the blastocyst, so that primitive amniotic folds appear to be growing centripetally over the embryo. If this interpretation is correct, this specimen is the most advanced of the group in terms of amnion formation.

The gross appearance of Specimen NE-39 illustrates the progressive cephalocaudal elongation of the disk, characteristic of this stage. Figures 16 and 17 represent sagittal sections through the embryonic area. Although the primary germ layers are well-established, there is no evidence of amniogenesis.

The chorionic vesicle of Specimen NE-55 extended the full length of the gravid horn. With the exception of NE-50, all other specimens in this age group occupied about two-thirds of the horn. At 10 microns, 105 serial sections were obtained, two of which are shown (Figures 18 and 19). In comparing these sections with those of NE-148 (Figures 13, 14, and 15), a marked similarity is noted.

PLATE 2

12. Mid-portion of 17-day-old blastocyst, NE-148, illustrating gross appearance of oval germinal disk and wrinkled trophoblast. 29×.

13, 14, and 15. Transverse 10-micron sections through NE-148. Numerous mitotic figures are seen among the tall columnar ectodermal cells of the disk. Evidence of a primitive groove in the caudal region is seen in Figure 15. 125×.

16 and 17. Representative sagittal sections of NE-39 illustrating cephalocaudal elongation of germ disk and well-defined primary germ layers. 125×.

18 and 19. Transverse 10-micron sections of NE-55. Note clear delineation of primary germ layers and well-defined separation of the mesoderm. The section in Figure 19 passes through the area of proliferation recognized as Hensen's node. 125×.

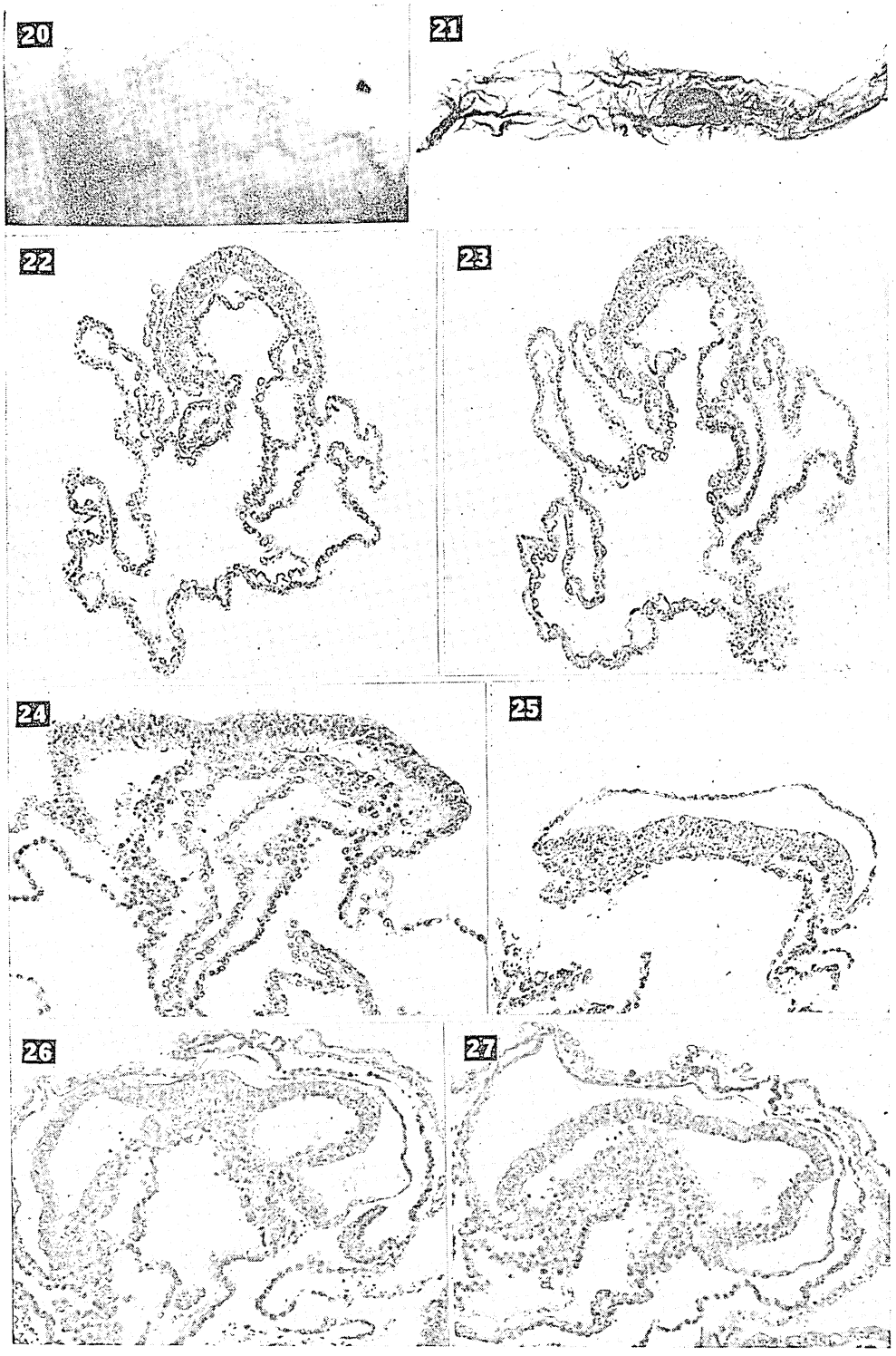


Plate 3

The former, however, appears to have progressed further, particularly with reference to a more pronounced Hensen's node and a more definitive mesoderm.

Eighteen-day embryos. Observations were made on five embryos recovered 18 days after the last breeding. In this age group, Specimen NE-49 appeared the least advanced. The embryonic disk (Figure 20) yielded 45 sections at 10 microns. These sections indicate that morphogenesis has not progressed appreciably beyond the stages previously described for 17-day embryos. The embryo displays a thickened ectoderm, a well-formed primitive streak and mesoderm, and continued extension of extraembryonic endoderm.

The gross appearance of Specimen NE-85 is seen (Figure 21). The embryo was located in the somewhat expanded mid-portion of the elongate, narrow chorionic vesicle. The embryonic disk measured 0.36 by 0.6 mm. after fixation, and 51 sections at 10 microns were obtained. Figures 22 and 23 are representative transverse sections through the embryo and vesicle, which show clearly the lateral portions of the mesoderm partially filling the area between the trophectoderm and yolk sac. The mesoderm has undergone delamination to form extraembryonic coelom. In the region where the heart will develop, this formation foreshadows the early appearance of pericardial coelom. As noted earlier in the 17-day-old Specimen NE-148, there is a suggestion of primitive amniotic folds. However, somatic mesoderm is not associated with the folded trophectoderm.

A definite primitive groove was grossly discernible in Specimen NE-90. This was confirmed in sections (Figure 24) with 56 somewhat oblique serial sections obtained at 10 microns. The continued elongation of the chorionic vesicle to fill the gravid horn and the progressive extension of the limits of the extraembryonic coelom placed this embryo in a stage of development beyond those previously described. Evidence for the upward bending of somatopleure at the periphery of the embryonic plate was entirely lacking, however.

When Specimen NE-37 was flushed from the horn of the uterus, examination of its entire length failed to produce a positive identification of the embryonic area. Representative portions of the central region were preserved, however, and in subsequent sectioning the embryo was located. Complete serial sections were not available for study, because the embryo had suffered some damage in

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PLATE 3

20 and 21. Gross appearance of 18-day-old germ disks of NE-49 and NE-85. Note progressive tendency for elongation of embryonic area. Chorionic vesicle remains slender, as seen in Figure 21. 25× and 15×.

22 and 23. Transverse sections of NE-85 (Figure 21) through the entire blastocyst in the embryonic area. Note the complete endodermal lining of the blastocyst, the lateral extensions of mesoderm, and the suggestion of early amniogenesis. 100×.

24. Oblique section through the primitive groove of NE-90. Note well-defined yolk sac and mesodermal elements. 125×.

25. A near-sagittal section of NE-37. Prominent features are the completed amnion, Hensen's node, primitive groove, and mass of notochordal cells. 100×.

26 and 27. Transverse sections of NE-131. The embryo is seen to be completely enclosed within the amniotic sac. Figure 26 is a section passing through Hensen's node; whereas, Figure 27 shows the thickening of the lateral mesoderm. 125×.

processing. The plane of the sections also passed somewhat obliquely through the longitudinal axis. The most notable feature of this embryo (as seen in Figure 25) is a well-defined, complete amniotic sac enfolding the developing embryo. Anterior to the thickened area of Hensen's node and the primitive groove is seen the rod-shaped mass of notochordal cells occupying the space between the ectoderm and endoderm.

Additional evidence for possible amnion formation during the 18th day of development was provided by Specimen NE-131. The embryonic area measured approximately 0.4 by 0.65 mm. after fixation, and a total of 74 transverse sections of 8 microns was obtained. This specimen was the most advanced in the series, judging by the microscopic appearance of the embryo and the extension of the chorionic vesicle into the nongravid horn. Figure 26 is a section through Henson's node showing the nodal area as a center of proliferation for a median band of cells, which seemingly merge with the hypoblast below. Fusion of the amniotic folds above the mid-dorsal region of the embryo completes the amniotic sac, and the embryo in its amnion lies free in the surrounding trophoderm. The thickened lateral mesoderm seen in this figure (and in Figure 27) foreshadows the emergence of the epimeric mesoderm and the future somatic mesoderm of the trunk area.

DISCUSSION

The determination of the exact age of recovered embryos is very difficult in most species, because of a lack of precise knowledge of the actual time of ovulation and the time of fertilization. The copulation age generally employed is, at best, an approximation and accounts for many of the discrepancies found in the literature. Thus, Winters *et al. (30)* dated the specimens from 6 P.M. of the day the cow was mated until the time of slaughter, and described a 13-day, 14-hr. embryo that was very similar to the 16-day-old specimen NE-93 of the present series. Chang *(5)* described similar embryos recovered from slaughterhouse material and, based on the description of Winters *et al. (30)* and Hamilton and Laing *(12)*, estimated their ages at from 13 to 14 days.

Similarly, the variation in development encountered within a single day among the specimens described in the present paper emphasizes the shortcomings of copulation-age estimates. This lack of precise information relative to the age of specimens makes it difficult to evaluate the influence of other factors, such as individuality, age, and breed differences.

Whereas the utimate aim of descriptive embryology is to characterize the successive and progressive developmental events as a continuum, the scarcity of accurately timed bovine material necessitates the establishment of standards within the limits of normal variation encountered using an arbitrary time-scale. The stage of embryonic development under discussion is one of extremely rapid growth and differentiation. In view of the heavy losses reported for repeat-breeders during the early stages of pregnancy, and the current interest in the causes of early embryonic mortality, suitable standards of normal bovine development expressed in terms of copulation age are needed.

The bilaminar condition of the growing germ disk described for the youngest specimen of the 16-day group is approximately comparable to the human embryo at the end of the second week *(17, 18)*, to that of the pig at about 8 or 9 days of age *(19)*, and to the sheep at from 10 to 11 days *(8)*. In the human, however, the amnion is established precociously by cavitation at from 7 to 8 days; whereas, in these other species, a more leisurely dorsal folding of the somatopleure occurs.

The more advanced specimens within the 16-day group are actively engaged in the gastrulation process. The growth and elongation of the blastocyst occurs soon after the endoderm has been established as a definite layer. The variation in the length of the chorionic vesicles noted in these specimens is often encountered within a single litter in the pig and attests to the rapidity of growth at this stage. Hawk *et al. (16)*, reporting on 31 bovine embryos recovered at 16 days, noted that the extraembryonic membranes varied in length from 2 to 225 mm., with a mean length of 95 mm. The disk itself lengthens cephalocaudally, converting the disk from a spherical to an oval shape. Both formative mesoderm and extraembryonic mesoderm make their appearance at this time, with the latter giving rise to the mesoderm of the extraembryonic somatopleure, splanchnopleure, and coelom. These features agree closely with the description by Winters *et al. (30)* of a 16-day, 14-hr.-old embryo.

The 17-day-old group is characterized by a continuing elongation of the blastocyst and local germ disk differentiation to establish the longitudinal axis of the embryo and the primitive streak. This stage of development is comparable to the 11- or 12-day-old pig, and the 13-day-old sheep, embryo. As might be expected, there is some overlapping in development when individual specimens are compared with the more advanced of the 16-day-old embryos, on the one hand, and the less developed of the 18-day-old specimens, on the other.

The most notable features of the 18-day-old group are the formation of the primitive groove, the extension of the boundaries of the mesoderm, the formation of the primitive node, the emergence of the notochord and, in some instances, the establishment of a definite amniotic sac. Although the successive steps in amnion formation are not clearly defined in these specimens, it is reasonable to assume a dorsal projection and fusion of anterior, lateral, and posterior amniotic folds similar to those found in the sheep and pig. With the attainment of the amnion and primitive streak stage, the 18-day-old cow embryo approximates the human embryo of the same age *(20)*, a 13- to 14-day-old sheep *(8)*, and a 13-day-old pig *(26)*. A suggestion that amnion formation in the cow is more precocious than in the pig derives from the contention of Heuser and Streeter *(19)*, that in the pig the embryonic area remains entirely exposed until after the primitive streak and neural folds are formed.

In the present series of 16 specimens, there are two instances of possible embryonic failure. Studies on the nature of reproductive failures in repeat-breeder cows have produced estimates that from 54 to 65% of all fertilized ova in such cows die before 34 days *(3, 4, 28, 29)*. In a similar study with a group of 80 repeat-breeder cows, Hawk *et al. (16)* estimated embryonic mortality be-

tween 16 and 34 days to be 51.7%, and suggested that the majority of the deaths occurred before 25 days.

It is undoubtedly significant that this period of high embryonic death coincides with these critical phases of morphogenesis during which the primary germinal tissues are established. These orderly, successive, integrated processes are dependent upon successful completion of each preceding step and a proper environment. Disturbing factors that in any way upset these normal interrelationships would preclude the successful continuation of the development of body form.

The expansion of the extraembryonic endoderm and the rapid growth of the trophoblast suggest that this period is a critical one in the nutrition of the embryo. The relatively late implantation (33 to 36 days) of the bovine embryo *(7, 22)* necessitates such a structural adaptation for the absorption of nutrients from the uterine environment after its own intrinsic energy sources have been exhausted. The functional state of the endometrium, in turn, depends upon maintenance of the corpus luteum, which at this same period in a normal estrous cycle would be undergoing degenerative processes. The stimulus for the continuation of a functional corpus luteum of pregnancy undoubtedly arises in the embryo and is probably of trophoblastic origin. Any consideration of possible causes of embryonic death, therefore, must take into account the complex interrelationships of the embryo, the uterus, and the gonads.

Then too, the numerous, intricate processes involved in gastrulation and primitive streak formation provide many opportunities for constitutional defects or fortuitous errors in development to express themselves in embryonic failures. These factors, in addition to the possibilities of endocrine and environmental causation of embryonic pathology, make accurate diagnosis relative to the nature and cause of individual failures virtually impossible with our present knowledge. A prime requisite for a final understanding of the problem is sufficient comprehensive information concerning the normal early embryology of the species to facilitate the recognition of abnormal and retarded development.

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INFLUENCE OF SEMINAL VESICULAR FLUID ON MORPHOLOGY OF BULL SPERMATOZOA[1]

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SUMMARY

This study presents the distribution of spermatozoa with protoplasmic droplets in the epididymides of slaughterhouse bulls. There were 11 paired and nine single epididymides included in the study. The mean per cent of spermatozoa with droplets in the caput, corpus, and cauda were 62, 41, and 84, respectively. The per cent of spermatozoa with droplets from the right and left sides of the reproductive tract of the same bull showed less variation than among bulls. Six per cent of the spermatozoa from the ampullae had droplets.

Fluids from the seminal vesicles, when added to epididymal spermatozoa, produced changes in the per cent of spermatozoa with protoplasmic droplets. The per cent of spermatozoa with distal protoplasmic droplets from the corpus and the cauda epididymis and the per cent of sperm with proximal droplets from the caput were decreased significantly ($P < 0.01$). The per cent of spermatozoa with distal droplets in the caput was increased significantly. The decrease in the per cent of total sperm with droplets after the addition of the seminal vesicular fluid was statistically significant ($P < 0.01$).

A round protoplasmic droplet is a structure normally observed on the epididymal spermatozoa of the bull. Attention was first drawn to this structure by Kölliker *(15)*, in 1856. Since then a number of workers have reported on its morphological origin *(6-10)*. The literature on the subject was summarized by McKenzie and Berliner in 1937 *(19)* and by Gresson in 1951 *(8)*. A majority of the reports agree that during spermatogenesis, after the elimination of the residual cytoplasm, a protoplasmic droplet, containing argentophil (Golgi) granules, is present immediately posterior to the head of the spermatozoon. Investigators have observed that spermatozoa after their entry into the epididymis possess the droplet in close proximity to the head, and that the droplet migrates along the mid-piece and the tail of the sperm during their passage through the epididymis and the ductus deferens *(3-5, 9, 12, 17, 22)*. Selivanova in 1937 *(22)* made a detailed study of the position of the droplet on spermatozoa from different parts of the reproductive tract of a number of farm animals. In two different parts of the caput of the bull she observed 23 to 67%, in the corpus 41 to 48%, and in the cauda 45 to 49%. She stated that the droplet shifted during the spermatozoon's passage from the caput into the cauda, and that the droplet was stretched along the mid-piece during the cell's sojourn in the corpus epididymis.

In 1947, Branton and Salisbury *(4)* also recorded the percentage of spermatozoa with droplets. They studied six bulls and obtained the following values for the spermatozoa with droplets: caput epididymis $80.32 \pm 6.79\%$, cauda

Received for publication May 14, 1957.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

69.90 ± 2.4%, and ampulla 23.19 ± 6.13%. Differences among bulls were not observed. Rao and Hart *(20)* gave similar percentages for cells taken from the cauda epididymis. Largerlöf *(16)*, from his extensive studies on impaired reproduction of bulls, concluded that the presence in the ejaculate of spermatozoa with droplets on their necks signified a reproductive disturbance. Hancock *(11)* has observed an unusually large number of sperm with droplets in infertile Guernsey bulls. Both Holt *(13)* and Mann *(18)* suggest that spermatozoa might use the droplet as a source of energy during the epididymal passage.

Normal ejaculates contain few spermatozoa with droplets *(1)*. A variety of solutions can cause detachment of the droplet, but the number of detachments is never as large as in normally ejaculated spermatozoa.

Selivanova *(22)* performed a large number of experiments in which she showed that seminal secretions and other solutions, as well as the rate of dilution, caused droplet detachment. Similar results were obtained by Rao and Hart *(20)*. Hancock *(12)* collected boar semen in separate 20-ml. fractions. In the first 20-ml. fraction, the number of sperm with the droplet was as high as in the ductus deferens. Subsequent fractions had a greatly reduced number of sperm with droplets. From this, Hancock concluded that the accessory gland secretions caused the detachment of droplets.

EXPERIMENTAL PROCEDURE

Bull reproductive tracts were obtained from the local meat-packing plant. The epididymides were separated from the testicles. There was some variation in lapsed time from the slaughter of the bulls until the different examinations were made. These examinations were usually completed within 2 to 3 hr. after slaughter. An incision was made in the middle of the caput, corpus, and cauda, and a small drop of semen was obtained by squeezing the epididymis near the incision, for studies on the distribution of sperm with droplets and on the position of the droplet of the spermatozoon. The caput incision was made on the side toward the corpus epididymis. Semen was obtained from the ampulla by stripping. Immediately, the semen was thoroughly mixed with eosin-nigrosin stain *(24)*. A uniform smear was prepared on a microscope slide. The slide was air-dried and the oil immersion objective was employed for observation. Three hundred spermatozoa were counted at random and the distribution of droplets recorded. The droplet was designated as proximal when situated on the neck and as distal when situated at the posterior end of the mid-piece of the sperm cell.

In another series of experiments, the influence of the fluids from seminal vesicles on the protoplasmic droplet was investigated. Seminal vesicular fluid was obtained by compressing the excised seminal vesicles and catching the fluid. On several occasions some admixture of blood was observed. The experimental drop of epididymal spermatozoa was placed in a small bacteriological culture tube to which 0.5 ml. of seminal vesicular fluid was added. Five minutes later, a drop of this mixture was stained with eosin-nigrosin and a differential count was performed as described previously. Control samples were treated in the same

manner as samples for the relative distribution studies. The significance of the changes caused by the seminal vesicular fluid was analyzed by the paired *t*-test; for the purpose of analysis all of the per cent values were converted to angles *(23)*.

RESULTS

In Tables 1 and 2, values recorded on the same line represent the caput, corpus, and cauda from the same epididymis. Some of the specimens were badly mutilated by the packinghouse workers so that some parts of the epididymis could not be used. This accounts for the missing values. The data in this study represent 11 paired epididymides and nine individual ones.

The distribution of droplets on spermatozoa from the different parts of the epididymides of bulls is shown (Table 1). Spermatozoa with droplets in the

TABLE 1

Percentage of spermatozoa with protoplasmic droplets and the position of the droplet as influenced by epididymal location

Epididymis number	Caput Epididymis		Corpus Epididymis		Cauda Epididymis	
	Distal droplet	Proximal droplet	Distal droplet	Proximal droplet	Proximal droplet	Distal droplet
	(%)					
1a[a]	9	25	14	0	92	0
1b	9	27	11	0	90	1
2a	7	24	20	0	84	1
2b	4	22	19	4	87	1
3a	0	85	37	1	79	3
3b	2	83	34	1	83	2
4a	36	30	48	1	76	1
4b	33	28	42	2	81	1
5a	27	30	69	3	81	1
5b	24	28	62	6	80	0
6a	6	56	43	2	81	2
6b	5	54	40	2	81	1
7a	14	52	38	0	82	5
7b	17	51	34	0	64	23
8a	22	44	39	5	90	0
8b	22	41	38	4	85	0
9a	7	39	40	4	73	2
9b	8	37	53	4	69	1
10a	0	80	25	0	79	2
10b	0	84	22	0	89	0
11a	9	61	29	0	....	....
11b	10	61	30	3	....	....
12	15	70	61	3	79	3
13	13	76	74	7	90	2
14	8	50	59	6	97	0
15	0	82	61	0	85	2
16	16	66	46	0	86	0
17	2	29	59	0	88	1
18	8	75	54	1	69	0
19	....	....	....	....	88	0
20	....	....	....	....	87	0
21	11	72	18	2	....	....
Range	0–36	22–85	11–74	0–7	64–97	0–23
Mean	9.3	52.4	40.0	1.0	83.2	1.0
Standard deviation	9.59	21.35	16.18	2.44	7.28	4.24

[a] The numbers 1a, 1b, etc. indicate these epididymides were from the same pair of testicles. A single number indicates that only one epididymis from a pair of testicles was used.

TABLE 2

Percentage of spermatozoa with protoplasmic droplets from various parts of the epididymides and the effect of seminal vesicular fluid on the droplet

Epididymis number[a]	Caput Epididymis				Corpus Epididymis				Cauda Epididymis			
	Position of droplet				Position of droplet				Position of droplet			
	Distal		Proximal		Distal		Proximal		Distal		Proximal	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
	(%)											
12	15	16	70	61	61	54	3	3	79	69	3	3
13	13	11	76	67	74	47	7	10	90	83	2	0
14	8	16	50	36	59	33	0	0	97	70	0	0
15	0	4	82	57	61	42	0	0	85	43	2	3
16	16	24	66	32	46	22	0	0	86	43	0	0
17	2	7	29	8	59	44	0	0	88	44	1	4
18	8	17	75	42	54	34	1	1	69	72	0	0
19	....	....	....	....	....	....	....	....	88	38	0	2
21	11	20	72	32	18	22	2	1	....	....	....	....
Mean	9.1	14.4	59.0	41.8	54.0	37.2	1.6	1.9	85.4	56.3	0.9	1.3
	$P < 0.01$		$P < 0.01$		$P < 0.01$				$P < 0.01$			

[a] The epididymis numbers correspond to the ones in Table 1.

caput epididymis comprised 61.7% of the total spermatozoa; this value is within the range of values quoted by Selivanova *(22)*, but is lower than the value of 80.32% observed by Branton and Salisbury *(4)*. Forty-one per cent in the corpus epididymis had droplets. This value corresponds to the value given by Selivanova *(22)*. The number of spermatozoa possessing droplets in the cauda was 84%, and this value is higher than the ones observed by other investigators. Branton and Salisbury *(4)* obtained the value of 62.9% and Rao and Hart *(20)* observed the range of from 18 to 75%. In the caput epididymis, spermatozoa with proximal droplets predominated; in the corpus and in the cauda epididymis spermatozoa with distal droplets predominated. These observations are in agreement with published reports *(4, 22)*.

The data (Table 2) show the influence of seminal vesicular fluid on the droplet position on spermatozoa from different parts of the epididymis. Addition of seminal vesicular fluid to spermatozoa from the caput caused a highly significant decrease ($P < 0.01$) in the per cent with proximal droplets. In the corpus and in the cauda epididymis the per cent of spermatozoa with distal droplets was decreased significantly ($P < 0.01$) by the addition of fluids. The addition of seminal vesicular fluids to sperm samples from the three parts of the epididymis caused a highly significant ($P < 0.01$) decrease in the total number of spermatozoa with droplets.

Ninety-four per cent of spermatozoa from nine ampullae had no droplets. The distribution of droplets on spermatozoa containing them was as follows: distal—from 1 to 19%, proximal—from 0 to 11%.

DISCUSSION

Differences between relative distribution values observed during this study and those observed by other investigators could stem from several sources. First, part of the difference could be accounted for by the inherent bull differences, though Branton and Salisbury *(4)* did not observe such differences in their study of six bulls. Second, the possible effect of the stain used on the droplet must be considered. This effect was not tested but, from other information available *(20, 22)*, one could infer that the stain might cause droplet translocation and disappearance. Third, no breeding records were available for these slaughterhouse bulls and the possibility of spermatogenic disturbances can not be discounted.

The values obtained for the paired sections (Table 1) show that within the same animal the two sides of the reproductive tract show similar distributions. This could indicate approximately the same maturation rate, or that the evacuations for the two sides were about equal.

The percentage of cells possessing the distal cytoplasmic droplet in the cauda was more uniform (range of from 64 to 97%) than the percentage of cells containing distal droplets (range of from 11 to 74%) in the corpus, or the proximal droplets in the caput (range of from 22 to 85%). It may represent a more uniform success of sectioning for the cauda than for the corpus or the caput, or that the spermatozoa from the cauda may respond more uniformly to the stain.

If the droplet is a cytoplasmic remnant containing Golgi granules, as the majority of investigators claim *(8-10)*, then droplets should be present in larger numbers on spermatozoa from the caput. Hancock *(11)* observed a progressive decrease in the number of droplets from the beginning to the end of the caput. It is unlikely that the droplet is acquired from epididymal secretions, although Gatenby and Collery *(6, 7)* have indicated that epididymal secretory granules can become attached to the spermatozoa. The number of epididymal spermatozoa without the droplet was largest in the corpus epididymis, rather than the cauda. This has been explained by Selivanova *(22)* as being the result of the stretching out of the droplet along the mid-piece. On occasion, thickening of the mid-piece was observed on spermatozoa from the corpus. Whether this was caused by stretching of the droplet can not be answered by this study.

Another important question raised by the presence of the droplet on spermatozoa is that of the maturity of the sperm. A number of authors *(11, 16, 21)* have stated that presence in an ejaculate of a number of spermatozoa with droplets indicates lowered fertility. Such statements are not wholly convincing, since the bulls under consideration produced ejaculates with a high count of abnormal sperm. Lagerlöf *(17)* modified his view and considered only spermatozoa with proximal droplets as possible indicators of sperm immaturity.

The absence of the droplet from ejaculated sperm is owing either to a mechanical or to a chemical factor, or both, operating during the transition from the epididymis into the ejaculate or the ampulla. The results (Table 2) show that the seminal vesicular fluid can cause a significant reduction in droplet number. In this respect, these data agree with the findings of Selivanova *(22)* and of Rao and Hart *(20)*.

Most of the spermatozoa found even in the first ejaculate come from the cauda *(2)*, a portion of the tract where droplets are numerous. Their absence in the ejaculate indicates a rapid disappearance of the droplet under the influence of ejaculation, rather than a rapid maturation, as suggested by some authors. Exhaustion trials *(2)* did not reveal increased numbers of sperm with droplets in consecutive ejaculates, and their results agree with the observation of Kirillov *(14)* in this respect.

In one occluded ductus deferens, the spermatozoa from the cauda were without droplets, which suggests a ripening process similar to the one described by Young *(25)*. On another occasion *(2)*, when ten successive ejaculates were taken from a bull, all the ejaculates had a high proportion of cells with droplets. Prior to this exhaustion, collections were taken from this bull approximately once a week. The usual influence of ejaculation must have been disturbed in this case, since on other occasions the ejaculates from this bull were free of spermatozoa with droplets.

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VALUE OF ADDING CORN SILAGE AND LIMITED QUANTITIES OF HAY TO A GRASS SILAGE, LIMITED GRAIN RATION FOR DAIRY HEIFERS[1]

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SUMMARY

Twelve Holstein and 12 Guernsey heifers were used to determine if dairy herd replacements can be raised satisfactorily from a few days to 2 yr. of age on a commercial milk replacement, 500 lb. concentrates and one or more of the following forage combinations: grass silage alone, grass silage and corn silage (50-50), grass silage and limited hay, and grass silage and corn silage (50-50) and limited hay. The silages were fed ad libitum and hay was fed at the rate of ¾ lb. per 100 lb. body weight per day. The inclusion of hay in the ration increased body weight gains, but the feeding of corn silage decreased gains. These results were due to differences in the consumption of nutrients from the various forage combinations. The Guernseys ate approximately the same total amount of forage as the Holsteins; thus, generally carried more body condition. The animals fed grass silage and hay averaged 94% of normal at 2 yr. The other groups did not make satisfactory gains. The feeding of corn silage had no effect on conception rate, but animals fed hay required more services per conception than those not fed hay. Groups of animals which made poor gains generally were delayed in becoming sexually mature and with-calf.

Because of the relatively large number of replacements that must be raised to maintain a dairy herd and to permit culling, anything that can be done to reduce the cost of feeding such animals is of considerable importance. The most promising way to do this appears to be to replace a maximum part of the concentrates in the ration with lower-cost nutrients from high-quality forage. With the increased use of grass silage as a means of obtaining early harvesting and low field losses owing to weather damage, it appeared that forage preserved in this way might be particularly useful in feeding dairy herd replacements when limited amounts of concentrates are used.

Sykes *et al. (8),* in an experiment with Holstein and Jersey heifers, fed limited milk and grain with high-quality forage in the following forms: alfalfa hay, alfalfa silage plus alfalfa hay, and alfalfa silage. They obtained normal growth with alfalfa hay but not with alfalfa silage fed as the sole forage. When a limited amount of hay was fed with the alfalfa silage, the growth rate was improved but did not equal that obtained on hay. In continuation of this work, Thomas *et al. (9)* found that the feeding of equal parts of corn silage and alfalfa silage did not increase gains over alfalfa silage alone. In these two studies, observations were made to 2 yr. of age and differences among groups receiving the

Received for publication November 1, 1957.

[1] Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 215. This study was supported by a research grant from the Charles H. Hood Dairy Foundation, Boston, Massachusetts. The authors appreciate the help of Mr. Guy W. Angell, Farm Superintendent, in growing and harvesting the forages used during the experiment.

various forages were not apparent until past 5 mo. of age. Porter and Kesler *(5, 6)* fed calves a limited amount of starter and either grass-legume silage or hay in one experiment and alfalfa silage or a comparable hay in another. These experiments, which continued until the calves were 112 days of age, did not reveal any difference between the silages and the hays.

Sherwood and Jones *(7)* reported that the extra gains in dairy heifers resulting from supplementing a ration of good-quality alfalfa hay with grain were small and did not justify the cost of the grain. Headley *(3)* fed all-roughage rations to older heifers and found that similar gains were obtained from alfalfa hay and alfalfa hay supplemented with alfalfa silage. Also working with older heifers, and limiting grain to 3 lb. per day, Wilson and Bartle *(10)* obtained only slightly lower growth rates when alfalfa silage was fed in comparison to comparable alfalfa hay. In earlier work at the New Hampshire Agricultural Experiment Station *(4)*, yearling heifers fed 2 lb. of grain per day made better gains when legume-grass silage was included in the ration than when comparable hay was fed. Converse and Wiseman *(2)* fed animals from birth through one lactation, using corn silage as the sole forage. Normal growth and development were obtained.

The study reported here was carried out to determine if an early-cut grass silage could be used successfully in feeding dairy heifers from a few days to 2 yr. of age, along with limited quantities of grain; also, to determine how the addition of corn silage and/or limited hay to such a ration would affect nutrient consumption and body weight gains.

EXPERIMENTAL PROCEDURE

Twelve Holstein and 12 Guernsey heifer calves purchased from nearby farms were used as experimental subjects. They were placed on the experiment at a few days of age. The calves were kept in individual pens and all feed was weighed. They were fed a commercial milk replacement according to the manufacturer's recommendations. Beginning as soon as the calves would eat it, a commercial 20% crude protein dry calf starter was fed at a level that the animals would clean up, until a maximum of 2½ lb. per day was being consumed. As each calf consumed a total of 200 lb. of this mixture, it was replaced abruptly by a 16% crude protein concentrate feed, mixed according to the New England Dairy Feed Conference formula for 1953, with the maximum daily concentrate consumption remaining at 2½ lb. per day. As the total grain consumption per animal approached 500 lb. (200 lb. starter and 300 lb. 16%), it was gradually decreased and discontinued at this amount. The age at which this happened usually was between 9 and 10 mo.

The animals were allowed access to trace-mineralized salt blocks in their stalls and a mixture of equal amounts of trace-mineralized salt and dicalcium phosphate in the exercise lot. They were permitted exercise as weather permitted. Irradiated yeast was fed to insure an adequate intake of vitamin D.

As the animals were placed on the experiment they were assigned to a particular forage combination by randomization. This resulted in three animals of

each breed being fed each of the following forage combinations: grass silage ad libitum, grass silage ad libitum and hay at the rate of ¾ lb. per 100 lb. body weight per day, grass silage and corn silage (50-50) ad libitum, and grass silage and corn silage (50-50) ad libitum and hay fed at the rate of ¾ lb. per 100 lb. body weight per day. The feeding of these forage combinations was started as soon as the calves would eat forage and continued through a period of 2 yr.

The grass silages used were early-cut mixtures of timothy, bromegrass, and native grasses containing relatively small proportions of Ladino clover and lesser amounts of other legumes. For the most part, the grass silages were preserved by the wilting method, but a few lots were preserved with sodium metabisulfite. Quality varied, as on most dairy farms. The corn silages fed were a fairly early-maturing variety harvested in the glaze stage. They could be classified as average in quality. The hays used were early-cut, field-baled mixtures similar in composition to the grass silages.

The heifers were bred artificially by the local technician, using semen selected by him. The breeding of the Guernseys was started on the first heat period after they had reached a body weight of 600 lb., or at the age of 18 mo., whichever came first. The comparable values for Holsteins were 750 lb. and 18 mo.

RESULTS AND DISCUSSION

The average body weights and heights at withers by 6-mo. periods for the animals receiving the various forage combinations are given (Table 1). These values also are given as a percentage of the Morrison normals. Table 2 gives the average forage consumption of the various groups by periods. Table 3 gives nutrient consumption from forage of the animals in the various groups in terms of crude protein and gross energy. These values were used because they are accurately and easily determined and eliminate the errors resulting from the drying of silage for analysis *(1)*. Table 4 shows a summary by periods of the effect of hay and corn silage supplements on body weight; whereas, Table 5 shows the effect of these supplements on protein and energy consumption.

Statistical analysis of the data for the 2-yr. period showed that there was a highly significant increase in growth when a limited amount of hay was added to the silage rations, and a highly significant decrease in growth when corn silage was added to the grass silage or grass silage plus hay rations. It further showed that the addition of hay increased energy consumption and that the feeding of corn silage decreased it. Both of these effects were highly significant. Similar effects on protein consumption were found.

When all of the data were combined, these effects amounted to increases of 16.0% in body weight gain, 20.3% in energy consumption, and 23.8% in protein consumption, owing to the addition of a limited amount of hay to the ration. When corn silage was added to the ration, the decreases amounted to 15.0% in body weight gains, 28.0% in energy consumption, and 30.5% in protein consumption. These results indicate simply that growth must be supported by adequate nutrient intake and that the nutrient intake was determined by the amount of the different forages that the heifers would eat. It does not indicate

TABLE 1

Average body weights and heights at withers and per cent of Morrison normal at end of each period

Forage combination	6 mo.				12 mo.				18 mo.				24 mo.			
	Body weight		Height at withers		Body weight		Height at withers		Body weight		Height at withers		Body weight		Height at withers	
	(lb.)	*(%)*	*(in.)*	*(%)*	*(lb.)*	*(%)*	*(in.)*	*(%)*	*(lb.)*	*(%)*	*(in.)*	*(%)*	*(lb.)*	*(%)*	*(in.)*	*(%)*
								Guernseys								
Grass silage	238	(89)	36.1	(97)	449	(92)	43.3	(100)	613	(93)	46.4	(100)	778	(95)	48.6	(101)
Grass silage + hay	235	(88)	36.3	(98)	448	(91)	43.5	(100)	620	(94)	46.8	(101)	797	(97)	49.3	(103)
Grass silage + corn silage	196	(73)	34.6	(93)	379	(77)	41.2	(95)	495	(75)	43.9	(95)	624	(76)	46.6	(97)
Grass silage + corn silage + hay	227	(85)	35.6	(96)	414	(85)	41.9	(97)	566	(85)	45.4	(98)	713	(87)	47.2	(98)
								Holsteins								
Grass silage	243	(64)	36.0	(91)	479	(70)	43.3	(94)	655	(74)	46.9	(94)	789	(72)	48.9	(94)
Grass silage + hay	295	(78)	37.3	(94)	566	(83)	45.0	(97)	828	(93)	48.6	(98)	1,006	(91)	50.5	(97)
Grass silage + corn silage	257	(68)	36.3	(91)	434	(63)	42.9	(93)	563	(63)	46.1	(93)	671	(61)	48.6	(94)
Grass silage + corn silage + hay	253	(67)	36.0	(91)	494	(72)	43.5	(94)	683	(77)	46.8	(94)	822	(75)	49.3	(95)

TABLE 2

Average forage consumption by periods

Forage combination	0–6 mo.			6–12 mo.			12–18 mo.			18–24 mo.			Total 24 mo.		
	Grass silage	Corn silage	Hay	Grass silage	Corn silage	Hay	Grass silage	Corn silage	Hay	Grass silage	Corn silage	Hay	Grass silage	Corn silage	Hay
	(lb.)														
	Guernseys														
Grass silage	1,142			4,355			7,848			8,936			22,281		
Grass silage + hay	893		132	3,569		410	6,658		690	7,640		908	18,760		2,140
Grass silage + corn silage	376	364		1,753	1,476		3,525	2,529		4,423	3,176		10,077	7,545	
Grass silage + corn silage + hay	442	374	103	1,859	1,717	402	3,552	2,650	622	4,381	3,188	867	10,234	7,929	1,994
	Holsteins														
Grass silage	973			4,786			7,862			9,156			22,777		
Grass silage + hay	1,032		178	4,236		478	7,430		904	8,197		1,202	20,895		2,762
Grass silage + corn silage	515	462		2,027	1,821		3,812	2,814		4,544	3,127		10,898	8,224	
Grass silage + corn silage + hay	379	367	149	1,525	1,303	448	3,216	2,650	781	3,997	2,119	962	9,117	6,439	2,340

TABLE 3

Average crude protein and gross energy consumption by periods

Forage combination	0-6 mo.		6–12 mo.		12–18 mo.		18–24 mo.		Total 24 mo.	
	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy
	(lb.)	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*
	Guernseys									
Grass silage	55	1,318	173	3,202	267	4,665	222	8,366	717	17,551
Grass silage + hay	58	1,299	178	3,612	302	5,237	258	8,813	796	18,961
Grass silage + corn silage	26	637	99	2,139	182	3,441	154	5,193	461	11,410
Grass silage + corn silage + hay	39	867	147	3,092	220	4,676	235	6,737	641	15,372
	Holsteins									
Grass silage	48	1,148	193	3,953	299	4,773	216	6,761	756	16,635
Grass silage + hay	70	1,548	218	4,502	363	6,229	294	8,738	945	21,017
Grass silage + corn silage	34	821	122	2,549	184	3,674	161	5,266	501	12,310
Grass silage + corn silage + hay	43	918	131	2,848	249	4,881	209	5,657	632	14,304

TABLE 4

Average body weights as influenced by hay and corn silage feeding

Forage additions	6 mo.		12 mo.		18 mo.		24 mo.	
	Body weight	Height at withers	Body weight	Height at withers	Body weight	Height at withers	Body weight	Height at withers
	(lb.)	*(in.)*	*(lb.)*	*(in.)*	*(lb.)*	*(in.)*	*(lb.)*	*(in.)*
				Guernseys				
Hay	231	36.0	431	42.7	588	46.1	746	48.3
No hay	217	35.4	414	42.3	554	45.2	701	47.6
Corn silage	212	35.1	397	41.6	531	44.7	685	46.9
No corn silage	236	36.2	448	43.4	616	46.6	786	49.0
				Holsteins				
Hay	274	36.7	530	44.2	756	47.7	914	49.9
No hay	250	36.1	457	43.1	609	46.5	730	48.8
Corn silage	255	36.2	464	43.2	623	46.5	746	49.0
No corn silage	269	36.7	523	44.2	742	47.8	898	49.7

that hay is a better feed than grass silage or that corn silage is an inferior feed. It means simply that animals fed under the conditions of this experiment had the preferences shown. These results are in good agreement with those of Sykes *et al. (8),* as far as the value of limited hay is concerned. The results from corn silage are not in agreement with the ideas generally held.

One of the other unexpected observations made during the experiment was that the Guernseys generally grew at a more nearly normal rate than the Holsteins. This is explained by the fact that the Guernseys ate about the same total amount of protein and energy as the Holsteins during the 2-yr. period, even though the Guernseys were smaller and grew less during the experimental period. These results suggest that, contrary to general opinion, the smaller breeds may grow at least as well on high-forage rations as do the heavy breeds.

Table 6 shows the reproductive performance of the animals on the various treatments. These results indicate a rather unsatisfactory breeding record. The lowest average age at conception for any group of Holsteins was 22 mo. and the lowest average age for Guernseys was 20 mo. The highest average age at conception was nearly 26 mo. for Holsteins and 24 mo. for Guernseys. In general, age at conception was related to growth. Those animals which made the poorest gains became pregnant last.

The average number of services per conception for the different breeds and forage combinations varied from 1.67 to 3.33. There was no difference between those animals which received corn silage and those which did not. On the other hand, those animals which received hay required 3.17 services per conception, whereas those which did not receive it required only 1.9 services per conception. This difference was significant at the 5% level. The reason for this apparently adverse effect of limited hay feeding on reproduction is not known.

No trouble was experienced in getting the calves to eat grass silage as early as they would eat hay. Corn silage was eaten a little more reluctantly. The feeding of silage did not cause scouring except for a period between 4 and 8 wk.

TABLE 5

Average consumption of crude protein and gross energy as influenced by hay and corn silage feeding

Forage additions	0–6 mo.		6–12 mo.		12–18 mo.		18–24 mo.		Total 24 mo.	
	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy
	(lb.)	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*	*(lb.)*	*(T.)*
					Guernseys					
Hay	98	2,166	324	6,704	522	9,913	493	15,549	1,437	34,332
No hay	81	1,955	272	5,342	449	8,105	376	13,559	1,178	28,961
Corn silage	65	1,504	246	5,232	403	8,117	389	11,930	1,103	26,783
No corn silage	114	2,617	351	6,814	569	9,901	480	17,178	1,514	36,510
					Holsteins					
Hay	113	2,466	348	7,350	612	11,110	503	14,395	1,576	35,321
No hay	81	1,970	315	6,502	483	8,447	377	12,028	1,256	28,947
Corn silage	76	1,739	253	5,397	433	8,554	370	10,923	1,132	26,613
No corn silage	118	2,697	411	8,455	663	11,003	510	15,499	1,702	37,654

TABLE 6

Reproductive performance of animals on different forage combinations

Forage combination	Age 1st heat	At first service		Services/ conception	At conception	
		Age	Weight		Age	Weight
	(yr.)(day)	*(yr.)(day)*	*(lb.)*	*(No.)*	*(yr.)(day)*	*(lb.)*
		Guernseys				
Grass silage	1 — 169	1 — 233	656	1.67	1 — 249	672
Grass silage + limited hay	1 — 199	1 — 215	647	3.33	1 — 305	702
Grass silage + corn silage	1 — 269	1 — 327	600	1.67	1 — 341	605
Grass silage + corn silage + limited hay	1 — 179	1 — 257	628	3.00	1 — 358	781
Av.	1 — 204	1 — 258	633	2.42	1 — 313	690
		Holsteins				
Grass silage	1 — 167	1 — 297	748	2.00	1 — 342	748
Grass silage + limited hay	1 — 136	1 — 198	824	3.00	1 — 305	929
Grass silage + corn silage	1 — 183	1 — 332	652	2.50	2 — 22	652
Grass cilage + corn silage + limited hay	1 — 208	1 — 275	734	3.33	2 — 51	869
Av.	1 — 173	1 — 275	734	2.73	1 — 360	813

of age. This may have been at a time when rumen activity had not reached a normal state. Later in the experiment, feces were normally rather dry. The feeding of grain to other animals fed these same forages resulted in feces of a much softer nature.

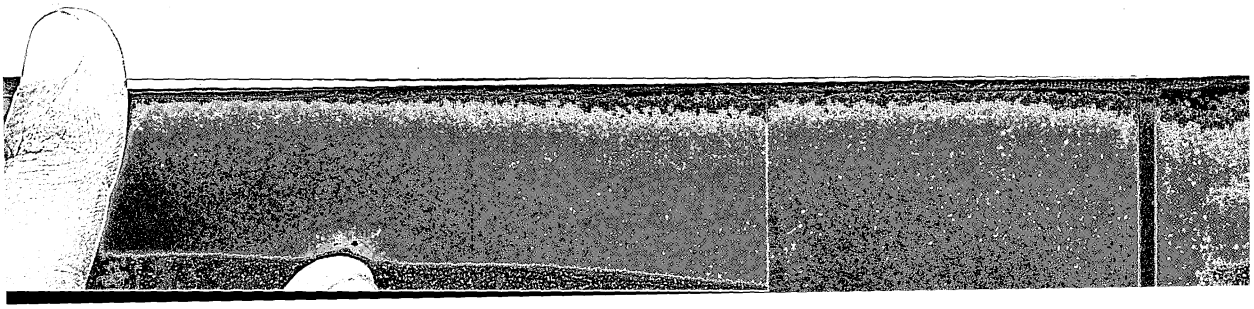
From a growth standpoint, this study indicates that rather satisfactory results can be obtained from feeding dairy heifers on 500 lb. grain and liberal quantities of early-cut grass silage supplemented with limited amounts of good hay. In fact, Guernseys fed in this way averaged 97% of the Morrison normal at 2 yr. and the Holsteins, 91%. From a breeding standpoint, however, the results were not satisfactory. Several extra months were required to get the animals into production. This would represent a considerable loss to the dairyman because of the increased number of animals which would be required to maintain a herd. The number of services per conception was considerably higher than desired, particularly in those groups which received hay. This seems rather odd, because these animals generally grew more rapidly than those animals which did not receive hay and were larger when bred. This problem should be given further study.

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TECHNICAL NOTE

FACTORS AFFECTING THE ESTROGENIC CONTENT OF ALFALFA SILAGE[1,2]

The occurrence of estrogens in plants has been the subject of a recent review *(2)*. Presumably, factors responsible for differences in the estrogenic content of plants, such as species of plant or stage of maturity *(4)*, would also influence the estrogenicity of silage made from plant material. Additionally, it has been reported that molasses preservation of alfalfa silage increases its estrogenic activity *(3)*. The object of this study was to determine the effect of stage of maturity, type of preservative, and length of fermentation on the estrogenicity of alfalfa silage.

All silages were made by placing 2 lb. of freshly chopped, second-cutting alfalfa in plastic bags *(7)* and allowing fermentation to progress for varying intervals. In some instances, a fresh alfalfa sample was dehydrated for determination of original estrogenic activity. The silages were made either without preservative or with the addition of 80 lb. of molasses or 8 lb. of sodium bisulfite per ton of plant material. The estrogenic content of the silages was determined, using a modification of a previously described technique for the assay of plant estrogens *(3)*. Five mice were used to assay each silage sample, except in one instance when four mice were used. In the latter case a fifth statistic equal to the mean of the group was added for ease of statistical treatment. All data were treated for statistical significance by the method of analysis of variance *(5)*.

The data presented (Table 1) indicate that alfalfa silage had a higher estrogenic content than the control laboratory ration. Alfalfa cut in the bud or 1/10th-bloom stage is more estrogenic than that cut at later stages of maturity. These differences were highly significant ($P < 0.01$) (Table 2). Statistical treatment of the data presented (Table 3) revealed no significant differences between the various groups, indicating that under the conditions of this experiment, type of preservation or ensiling per se was without effect. This is in contrast to work previously reported *(3)*. It has been our experience that molasses preservation has a variable effect on the estrogen content of alfalfa silage.

Recent work indicates that strains of alfalfa vary widely in their natural content of estrogenic compounds *(6)*. One such compound isolated from alfalfa is coumestrol *(1)*. It is

[1] Journal Paper No. 1148 of the Purdue University Agricultural Experiment Station.

[2] This project was supported in part by a grant from the Commercial Solvents Corporation, Terre Haute, Indiana.

TABLE 1

Effect of stage of maturity and type of preservative on estrogen content of alfalfa silage

Stage of maturity	Type of preservative	Body wt.	Uterine wt.	Body wt.
		(g.)	*(mg.)*	*(%)*
Bud	None	26.40	18.68	0.071
	Molasses	23.20	36.80	0.159
	Sodium bisulfite	24.60	26.16	0.107
Mean			27.21	
1/10th-Bloom	None	25.60	31.44	0.123
	Molasses	25.60	26.72	0.105
	Sodium bisulfite	26.20	35.88	0.136
Mean			31.35	
1/2-Bloom	None	23.00	15.60	0.067
	Molasses	24.20	19.20	0.079
	Sodium bisulfite	24.60	20.92	0.086
Mean			18.57	
Full-bloom	None	24.60	22.68	0.092
	Molasses	24.25	18.10	0.075
	Sodium bisulfite	25.60	24.88	0.098
Mean			21.89	
Mature	None	26.00	25.04	0.097
	Molasses	28.20	22.60	0.081
	Sodium bisulfite	26.00	20.60	0.080
Mean			22.75	
Control—laboratory ration		26.20	14.44	0.055

concluded that the estrogenic activity of alfalfa silage is more likely to be a function of original activity of the alfalfa than a result of the ensiling process.

ACKNOWLEDGMENT

The authors are grateful to Dr. M. W. Carter for his assistance in the statistical treatment of the data.

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TABLE 2

Analysis of variance. Stage of maturity and type of preservative

Source	D. F.	Sum of squares	Mean square	F-ratio
Total	79	4,627.26		
Treatment	15	3,229.84	215.32	9.86[a]
Control vs. others	1	460.66	460.66	21.10[a]
Stage	4	1,487.41	371.85	17.03[a]
Preservative	2	116.60	58.30	2.67
Preservative × stage	8	1,165.18	145.65	6.67[a]
Error	64	1,397.42	21.83	

[a] $P < 0.01$.

TABLE 3

Effect of length of fermentation and type of preservative on estrogen content of alfalfa silage

Length of fermentation	Type of preservative	pH	Body wt.	Uterine wt.	Body wt.
(days)			*(g)*	*(mg.)*	*(%)*
	None	5.2	27.00	24.62	0.092
2	Molasses	4.6	24.20	22.52	0.093
	Sodium bisulfite	5.6	28.20	23.72	0.084
	None	4.6	25.60	22.76	0.089
4	Molasses	4.3	29.00	24.60	0.085
	Sodium bisulfite	5.5	27.40	23.20	0.084
	None	4.8	26.20	24.76	0.095
8	Molasses	4.2	25.80	24.68	0.096
	Sodium bisulfite	5.4	26.80	23.36	0.088
	None	4.6	25.60	23.56	0.090
16	Molasses	4.2	25.60	27.24	0.107
	Sodium bisulfite	5.4	26.00	20.80	0.081
	None	4.6	27.80	22.84	0.082
32	Molasses	4.2	25.60	23.84	0.094
	Sodium bisulfite	4.9	25.20	21.72	0.087
	None	4.5	24.20	24.52	0.103
64	Molasses	4.2	27.60	25.04	0.092
	Sodium bisulfite	4.5	25.20	22.96	0.090
	None	4.7	23.20	21.28	0.092
128	Molasses	4.5	23.20	22.80	0.099
	Sodium bisulfite	4.5	24.40	21.04	0.087
Control—dehydrated alfalfa			27.00	24.92	0.094

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SOLIDS-NOT-FAT IN MILK SYMPOSIUM

This symposium was sponsored by the A.D.S.A. Dairy Cattle Breeding Committee, a joint committee of the Production and Extension Sections. Its purpose was to summarize research on the solids-not-fat and other components in milk, and to exchange ideas.

The discussion that followed the presentation of papers indicated a need for an extended study on farm herds throughout the country. To bring this about, the Production and Extension Sections requested that the A.D.S.A. Dairy Cattle Breeding Committee appoint a committee to develop a cooperative research program among the various experiment stations. The committee appointed was: D. Madden, Michigan State Univ., East Lansing, *Chairman;* J. E. Legates, North Carolina State College, Raleigh; W. J. Tyler, Univ. of Wisconsin, Madison, and S. N. Gaunt, Univ. of Massachusetts, Amherst. The committee has contacted experiment stations throughout the country to ascertain their interest in cooperating in the project and over 20 states have indicated an interest. Details of agreements, standardization of procedures, equipment, data collection, and methods of analysis are being studied.

—S. N. Gaunt, *Chairman, A.D.S.A. Dairy Cattle Breeding Committee*

NONGENETIC FACTORS AFFECTING THE PRODUCTION OF NONFAT MILK SOLIDS BY THE BOVINE[1]

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Factors that affect the composition and total production of milk by the bovine include: breed, individuality, nutrition, locality, bioclimatological effects, management, stage of lactation, and physiological factors (age, exercise, estrus, gestation, yield, sickness, etc.). Many of these factors are inter-related and for some the effect may be small, except under exceptional conditions. The major factors affecting the production of nonfat milk solids on which there has been considerable work are breed, individuality, nutrition, bioclimatological effects, and stage of lactation. Breed and individuality are more properly thought of as genetic factors. An attempt will be made here to review briefly some of the knowledge pertaining to bioclimatological and nutritional effects; then to conclude with some studies concerning protein production and the stage of lactation.

It is important to consider, however, some concepts regarding the nonfat solids of milk. Careful judgment is necessary in the current economic switch in emphasis from butterfat to the nonfat solids. It is well known that cow's milk is a nutritionally valuable product, in that it was designed by nature to supply nourishment for the young animal. That it fulfills this function and is also advantageous for the growing and adult animal is supported by chemical analyses, which show that milk contains a favorable balance of fat, carbohydrate, and protein fortified with minerals and vitamins *(7)*. However, in the light of current knowledge, the relative importance of each of the constituents should be appreciated. Due to the availability from other sources of many of the nutrients present in milk it appears that in the future protein will be the constituent which justifies milk nutritionally and, as nutritional awareness increases, most likely it will be the component which will justify milk economically. We are living in an age where a nutritional analysis of contents is appearing even on the labels on cans of dog food. World-wide, there is a shortage of food calories as such *(18)*. However, there is a greater shortage of high biological-value proteins *(20)*. The ability of the dairy cow as a ruminant to convert low-grade roughage and nitrogen sources into high biological-value protein is perhaps its most important asset.

Lactose (milk sugar) is a source of energy; however, glucose is available much less expensively from other sources; also, there is no biochemical evidence that preformed lactose or the galactose portion of lactose is essential nutritionally even for the new-born *(7)*. The small amount of galactose required for the developing nervous tissue can be supplied amply by the Waldenase enzyme system present, which interconverts glucose and galactose *(7)*. By this same mechanism, the galactose portion of lactose is converted to glucose, where it can be utilized by the animal. It is well known that a good portion of the consumption of calcium and phosphorus of the American people is from milk. However, this in no way justifies milk,

[1] Presented at 52nd Annual Meeting of the American Dairy Science Association, Oklahoma State University, Stillwater.

since if it did, enterprising enterpreneurs would soon supply the market with more direct and less expensive sources of calcium and phosphorus than milk. Thus, caution is necessary in not putting too much weight on the total nonfat solids but in giving consideration to the relative importance of each of the constituents. Since it is believed that protein is the most important constituent, the emphasis in this discussion will be devoted to the milk proteins.

It is pertinent to point out the paradoxical situation which arises when all of the nonfat solids are considered to be of equal value. On a basis of total nonfat solids present in milk per se, a good argument can be made in favor of the Holstein breed over the breeds producing higher fat. Certainly, Holsteins produce milk containing more of the caloric content present in the nonfat solids relative to the butterfat *(11)*. However, in producing more milk with less butterfat, the Holstein also is producing more total lactose in that milk. If one is to be fair, the caloric contribution of fat plus lactose should be compared to that of protein. On this basis, it is hard to find much difference between the common breeds in their protein-producing abilities. It must be recognized, however, that large differences do exist between individual animals.

Specific factors affecting the nonfat solids milk production. *a*) Bioclimatological effects—The fact that a decrease occurs in the fat and nonfat solids composition, as well as in the total production of milk during the summer months, has been well established. However, in analyzing some of the older studies, which show large compositional decreases during the summer, it is apparent that many of them were herds in areas where there was a question of nutritional adequacy in the summer pastures or where there was a tendency for more cows to freshen at one time of the year, thereby introducing a stage-of-lactation effect. Various investigators have made excellent reviews of the literature concerning some of these aspects *(5, 12)*.

In dry years, there is a tendency for the total yield of milk to decrease, along with a decrease in the nonfat solids composition and an increase in the fat composition. In wet years, there is a tendency for both the nonfat solids and the fat percentage to decrease, with or without an increase in the pounds of milk, depending on the level of nutrition. These effects are not as important to consider today, when dairy cows are not allowed to shift for themselves on range and pasture as they used to, and when adequate nutritional feeds and supplements can be made available at all times.

The effects of bioclimatological factors on milk production have undergone considerable scrutiny in recent years, with emphasis on the programs for developing suitable cattle breeds and strains for different climates. Studies of the Missouri investigators indicate that the decrease in feed consumption at higher temperatures is apparently the major reason for decreased milk production *(3)*. The water consumption of high-producing cows decreases and of low producers increases. Such changes start in the range of from 70 to 85° F. for the common North European breeds. A rise in fat and total solids composition of the milk occurred above 80-90° F., varying somewhat with the breed and individual *(4)*. Various workers *(2, 13)* have correlated the increase in body temperature with the decrease in feed consumption and in total milk production. Studies at the more northern experiment stations have shown that the common breeds are most resistant to cold temperatures than was previously thought, and that milk production and composition do not suffer.

b) Nutritional effects—Many of the reports in the older literature describing bioclimatological effects were actually describing effects indirectly caused by the nutritive feed potential available to the lactating cow *(12, 15)*. A high-producing cow needs a smaller percentage of its feed intake for maintenance and produces relatively more milk. This milk tends to be of a lower fat composition, but contains more energy present as protein and lactose.

It is not the idea here to discuss dairy cattle nutrition. However, one can point out some principles concerning milk produced by cows, either in starvation or in overabundance of feeding. The composition of the individual protein constituents of milk in either case is probably not altered materially, although the relative composition may be. For example, whether starved or well-fed, cows produce milk containing the same amino acid composition in the α-casein; in starvation there is just less of it. Proteins fed to the cow do not appear in the milk. This is also true of the sugars, for if sugars different from those appearing in milk are fed, they do not appear in the milk. An exception to this is butterfat. If certain long-chain fatty acids are fed they may appear in the milk as part of the butterfat. The same is true of certain minerals also, but not of others.

Many of the reports in the older literature relating to effects of locality are today directly traceable to nutritional shortages. Reid *(15)* has made an excellent report on these as well as other aspects. The effects on total production of excess molybdenum, selenium, and zinc in the diet are apparent, as are the effects of deficiencies, such as iodine or cobalt. Today, through proper nutritional understanding, adjustments can be made correctly to allocate the dietary intake of these materials.

c) Stage of lactation—This is probably one of the largest factors contributing to daily and monthly variations in the nonfat solids production and protein composition of milk of the individual cow. An appreciation of it is necessary in considering the productive capacity of an individual cow during the entire lactation.

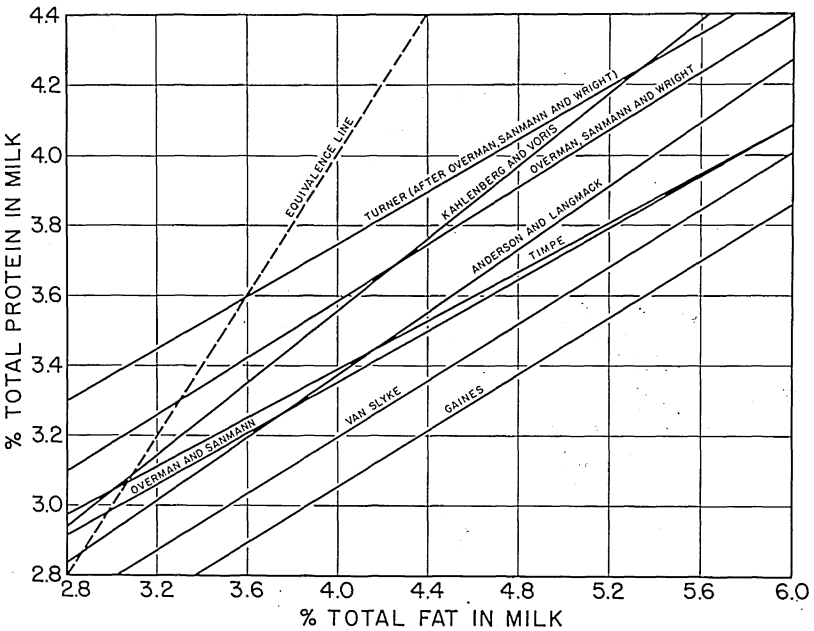


FIG. 1. Relationship between the protein and fat content of milk as found by several investigators. [Table taken partially from McDowall *(12)*, where the references not quoted in the text may be found. Some may be found also in reference *(11)*.]

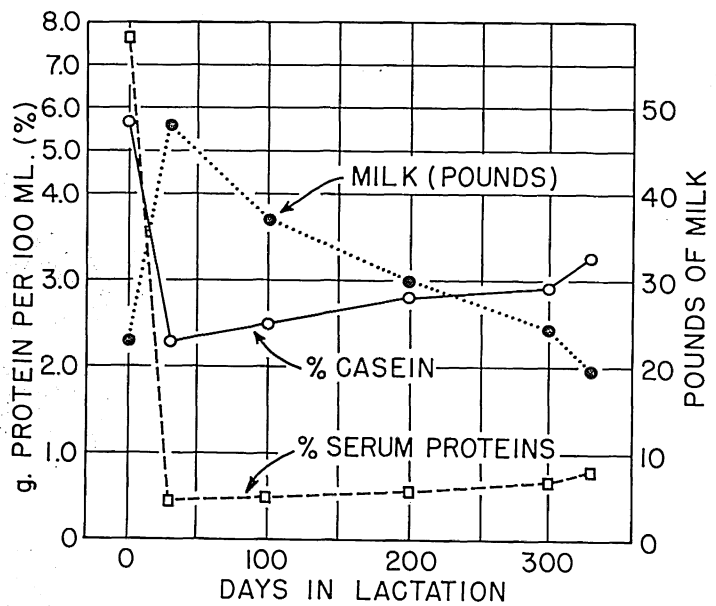


FIG. 2. Effect of stage of lactation on the total casein and whey protein composition of milk.

Large day-to-day fluctuations occur in the total butterfat yield (in per cent composition, but more so in total yield per day) *(11)*. Because the percentage of lactose is quite constant in milk *(14)*, its daily production varies with the pounds of milk produced. Total protein yield, however, tends to be more constant day to day *(1, 11)*.

Large changes occur in the ratio of protein to fat during the lactation period, varying considerably with the breed and individual *(11)*. Starting at about 2.0 at parturition, the ratio decreases to about 0.7 at the time of maximum milk yield, increases slowly to about 0.95 at 300 days, and then decreases to about 0.80 at 330 days if the cow is not dried off. Such differences point up only one of the reasons why such widely varying relationships between

the fat and protein content of milk have been found. Some of these relationships are shown (Figure 1). Analysis of these relationships found by various investigators shows that there is up to a 30% discrepancy between the highest and the lowest correlation lines. It is interesting to note that the relationship *(19)* most commonly quoted or reproduced *(5)* does not agree with the authors' data from which it was constructed *(14)*, and is the line farthest away from the others. Even though the positive correlation indicates that a high-fat milk is higher in protein also, it should be noted that it is not equivalently higher, because for each increment of more protein a greater amout of fat is present. On a caloric basis (nine and four calories per gram for fat and protein, respectively), the difference is even more striking. The reasons for such discrepancies in the relationships are apparent when breed, stage of lactation, nutritional, and other effects were not taken into proper consideration. The dangers in assuming per se that breeding for high-fat yield leads also to high-protein yield also are apparent.

During the lactation period, definite changes occur in the protein composition of milk *(11)*. The average casein and whey protein compositions of milk are high at parturition, reach a minimum at the time of maximum milk yield, and then increase to the end of lactation. This is illustrated (Figure 2). In addition to the changes in the gross protein composition of the milk, the curves for total protein production also show characteristic trends. This is true also of the individual protein components *(10, 11)*.

At this point, it is necessary to review some facts concerning the major protein components of milk. α-, β-, and γ-Casein are the major proteins of casein, and the immune globulins, α-lactalbumin, β-lactoglobulin, serum albumin, and some other minor identified and unidentified proteins comprise the whey proteins. The immune globulin fraction, which is highest in colostrum but also present in normal milk, composes essentially the classical "lactoglobulin" fraction; β-lactoglobulin is the chief component of the classical "lactalbumin" fraction. This nomenclature has undergone revision in recent years and today the terms "lactoglobulin" and "lactalbumin" are outmoded *(8)*. Instead, the names of the individual proteins comprising them, with the suitable prefixes, should be used.

These individual proteins possess different properties and arise from two different origins. Experiments conducted in this laboratory *(9)* with C^{14} have indicated that the four proteins, α-casein, β-casein, α-lactalbumin, and β-lactoglobulin, are proteins synthesized from a free amino acid pool in the secretory cells of the mammary gland. γ-Casein, the immune globulin, and milk serum albumin apparently are proteins which enter the gland and milk preformed from the blood stream.

The production of proteins synthesized in the secretory cells of the mammary gland in most cows tends to reach a maximum within a week after parturition, then declines to the end of lactation, with a slight increase shown at the time of maximum milk yield *(10)*. In contrast, like the production of total milk, the daily yields of fat and lactose tend to reach a maximum together about a month after parturition. The relative compositions of the proteins not synthesized in the gland but coming from the blood are high at parturition, reach a minimum at the time of maximum milk yield, then start increasing to the end of the lactation period *(10)*. However, the total production of these proteins keeps decreasing through the lactation, but not quite as rapidly as the proteins synthesized in the gland.

Unfortunately, with the economic spotlight on fat for so many generations, much knowledge is lacking concerning the protein-producing abilities of the dairy cow, but we have attempted to fill in a little of that gap.

Man has made great strides in controlling his environment and that of his stock animals. Many factors important years ago are now being controlled through scientific understanding of their principles. This is especially true of many bioclimatological and nutritional factors. The elucidation of the biochemical mechanisms by which physiological and nutritional factors mediate milk production, and how they can be controlled to further man's desires, await greater understanding.

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ASSAY FOR THE SOLIDS-NOT-FAT OF COW'S MILK[1,2]

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The recently revived interest in the solids-not-fat (SNF) content of milk has been stimulated by *1*) the current surplus of fats, *2*) calorie-conscious milk consumers, and *3*) a recognition of the presence of high-quality proteins in this portion of the milk. Ultimately, these factors may bring forth a greater effort on the part of dairy cattle breeders to increase the SNF content of milk, along with a more realistic milk-pricing plan designed to recognize the real value of SNF. Such plans, in varied versions, are being employed presently in a few manufacturing areas. Despite the apparent selling points, we must recognize that such pricing schemes have little merit in a fluid milk market. It should be stated here that the present situation of surplus and subsidy is not conducive to a change in the pricing structure. However, it is conceivable that the present pattern will be altered in years to come if high-quality proteins are forced into a competitive market. In such a market we must be ready to launch into a breeding and testing prgram geared to the SNF portion of milk.

Laboratory methods for estimating the major components of the SNF fraction—moisture, proteins, lactose, and ash—are well established. Today, our primary emphasis is being directed toward the development of simple but accurate procedures suitable for use in a field-testing program, such as the present set-up found in the DHIA program. The components most likely to come under consideration for direct measurements are SNF, T.S. (moisture), and/or proteins. Methods for estimating lactose and ash are relegated to the laboratory situation.

The methods seemingly most suitable for the routine estimation of SNF components are discussed:

Specific gravity determinations. Hydrometric techniques have been investigated extensively and offer a practical approach to the estimation of the major components of milk. The most promising techniques are:

Lactometers. 1) The Quevenne lactometer, read at 60° F. in milk previously warmed to 105° F. and immediately cooled, furnishes specific gravity readings suitable for substitution in the estimation equation T.S. (%)= 0.25 L + 1.2 × F (%) to give values for individual cow's-milk samples approximating gravimetric determinations *(4)*.

2) The Watson-pattern lactometer *(23)*, designed to be read at 102° F., has received considerable attention over the past four years. In the laboratory, where water bath temperatures can be maintained at 102° F., this lactometer along with the estimation equation

$$\text{T. S. (\%)} = 1.33\text{F (\%)} + \frac{273\text{L}}{\text{L} + 1000} - 0.40$$ has proven satisfactory. A recently designed instrument, measuring only 6 in. over-all and graduated from 26 to 33 lactometer degrees, requiring but 4 oz. of sample, has been laboratory-tested *(17)*. This instrument is being "field-

[1] Presented at the Solids-not-fat Symposium, A.D.S.A., Oklahoma State University, Stillwater, June 28, 1957.

[2] Approved for publication as Journal Article 2210 from the Michigan Agricultural Experiment Station, East Lansing, Michigan.

tested" with encouraging results. Minor modifications in its design are recommended.

3) The Dutch-pattern Quevenne lactometer *(22)*, designed to read at 68° F. and measuring 8 in. over-all, requiring only 4 oz. of sample, has been employed with the estimation equation $\text{T.S.}(\%) = 1.23\text{F} + \frac{260\,(\text{L} + 1.8)}{\text{L} + 1000}$. Recent studies made at this laboratory indicate that this lactometer and/or its estimation equation is not as satisfactory for measuring the T.S. of individual cow's milk samples as other lactometric techniques *(17)*.

Westphal balance. Specific gravity determinations are made by a balancing technique obviously too tedious for the degree of accuracy achieved *(10)*.

Subsurface hydrometer. This instrument is being developed at Washington State by Golding *(9)* and is based on the same hydrometric principle as the lactometer. A set of plastic disks of varying densities and arranged on a stainless steel rod is placed in a tube of milk and the entire assembly is centrifuged in a Babcock centrifuge. The suspended disk, marking the dividing point between the floating pack and the bottom cluster, corresponds to the density of the skimmilk portion of the milk. This value is relative to lactometer degrees and is applied in estimation equations for the T.S. or SNF values. The technique is ingenious and can be used to measure the specific gravity of the skimmilk portion. To demonstrate its practicability, the instrument should be field-tested.

Falling drop. Barbour and Hamilton *(5)* devised a technique for measuring the specific gravity of a drop of blood by comparing its falling time to the falling time of a standard salt solution in an immiscible liquid. This interesting technique, applied to milk, is being studied in our laboratory. Preliminary results are encouraging. If the method proves to be reliable, it will incorporate the advantages of speed and small sample size. A potential application exists in its use by a central testing laboratory.

All hydrometric methods are subject to the general criticism that relatively accurate density values must be substituted into estimation equations developed from mass data. The milk samples of nonaverage or abnormal individuals, not fitting such an equation, are evaluated erroneously. This weakness can be modified somewhat, and the estimation equations made more sensitive, by deriving specific estimation equations for the herd and/or area under test.

Gravimetric determination of total solids. Direct measurements of total solids, by determining the residue weight following the removal of moisture by heat, constitute the basis for the most reliable measuring techniques.

Method of the Association of Official Agricultural Chemists. This oven-drying technique requires three painstaking, analytical weighings and several hours of oven-time *(3)*. Although an accepted standard for moisture (T.S.) determinations, the method requires equipment much too elaborate and sensitive for field conditions. Large numbers of samples present a real problem unless analyzed under highly organized laboratory conditions.

The Mojonnier modification. Moisture (T.S.) determinations have been accelerated considerably by the use of a vacuum oven *(18)*. In recent years, various modifications of the vacuum oven and changes in operating procedures have made this method even faster *(15)*.

Short-cut drying and weighing techniques. a) Infra-red lamps employed as a source of heat and in combination with torsion wire balances have succeeded to a degree in making the dry residue–weight technique a field method *(6)*. Unfortunately, these techniques are for single sample determinations and have not proved successful under conditions encountered in the field.

The oxidometric method. A ceric sulfate method recently reported claims a high correlation with gravimetric determinations *(16)*. Some difficulty with the rather indefinite and unstable endpoint of the reaction mixture has been experienced. Until this tricky endpoint is mastered, the technique does not appear to have field possibilities, although the manipulations involved are but simple titrations.

Protein measurements. Many dairy people believe that the proteins of milk are its primary asset and should serve as the basis for measurement and evaluation. The standard procedures for protein determinations are laboratory techniques based on the Kjeldahl method and obviously are too involved to be used as a field method or for large numbers of samples. However, rapid and fairly simple methods for the estimation of the protein content of milk have been used with some success. A few of these methods possess possibilities as field methods.

Formol titration. The formol titration is an old friend of the protein chemist and has been adapted to the estimation of protein for many purposes. In fact, many of the dairy plants in Europe utilize the formol technique to measure the protein content of patrons' milk *(8)*. The procedure reported by Richardson *et al. (20)* at Oregon State has possibilities of becoming a satisfactory field test for the estimation of the protein content of milk. This method consists of a simple titration utilizing a standard sodium hydroxide solution to the phenolphthalein endpoint before and following the addition of a small quantity of formalin.

Titration to pH 4. The buffering capacity of milk, owing to proteins and salts, is utilized to estimate its protein content by a procedure developed at Washington State by Ashworth *et al. (1)*. The method has laboratory possibilities as a method capable of analyzing large numbers of samples, but does not appear to be suitable for field testing because of the equipment and techniques involved.

Dye binding. A rapid, simple procedure for measuring milk proteins has been reported by Ashworth and Seals at Washington State *(2)*. The test is based upon the ability of the disulfonic acid dye, Orange *G*, to bind with the basic groups of the protein molecule. The test has given satisfactory results and appears to have the potentiality of becoming a method suitable for the routine laboratory analysis of normal milk samples.

Protein components. Methods available for estimating the protein complement of milk are laboratory procedures represented by techniques such as electrophoresis, chromatography, and solubility. Because of the complexity and time requirements of these techniques, it is doubtful that large-scale evaluations could be undertaken *(21)*.

Prediction equations. Derivative equations, obtained from mass data, offer a practical means of estimating indirectly the SNF or T.S. content of milk from fat, protein, or other highly correlated component relationship. There are inherent errors associated with this technique which must be recognized. Most prominent among these is that the prediction equations represent mean relationships; therefore, they do not predict accurately at the extremes *(11, 12, 13, 14, 19)*. Whether this constitutes a practical objection is debatable.

Other methods. Additional methods for the determination of milk solids, as studied by Davey and Patton *(7)*, include chemical oxygen demand, hydrolysis of acetyl chloride in the presence of pyridine by water, Karl Fischer reagent, modified drying procedures, and others.

Conclusions

The small Watson-pattern lactometer offers a method sufficiently accurate and simple to use in large-scale testing programs, either in a laboratory or in field application.

The formol titration appears to be best suited as a rapid and simple method for the estimation of the protein content of milk under field conditions.

A recently developed technique utilizing the protein-binding capacity of Orange *G* might possibly offer a practical method for measuring the protein of milk at the laboratory level.

More sensitive estimation equations for T.S. and SNF from lactometer readings, and corresponding prediction equations for estimating highly correlated components, can be derived from mass data obtained under local conditions.

The direct determination of T.S. of SNF by sensitive gravimetric techniques remains in the realm of laboratory procedure.

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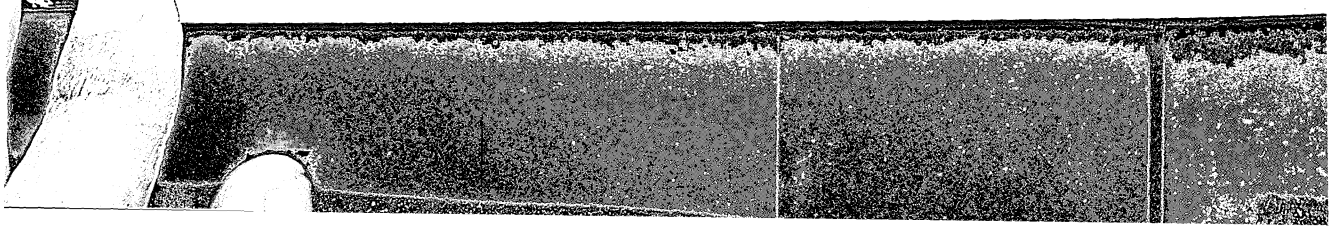
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GENETIC ASPECTS OF SOLIDS-NOT-FAT AND ITS COMPONENTS [1]

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The evidence for genetic variation in the composition of milk may be divided into three sources: (a) Breed differences, (b) sire differances, and (c) individual differences.

Breed differences. The fact that differences exist among breeds in the composition of milk has been known for some time. A comprehensive study of this problem was made by Overman and coworkers *(11, 12)* and a table prepared by Turner *(19)* from these data has received wide publication in many of our dairy science textbooks. Figures from this table are given (Table 1).

TABLE 1

Breed differences in composition of milk

Breed	Component of milk					
	Fat	Protein	Lactose	Ash	T.S.	S.N.F.
Ayrshire	4.00	3.53	4.67	0.68	12.90	8.90
Swiss	4.01	3.61	5.04	0.73	13.41	9.40
Guernsey	4.95	3.91	4.93	0.74	14.61	9.66
Holstein	3.40	3.32	4.87	0.68	12.26	8.86
Jersey	5.37	3.92	4.93	0.71	14.91	9.54

Bailey *(1)*, in a recent review article, has pointed out that there appear to be strain or herd differences in milk composition. This seems to be important, because research by many workers has indicated that the figures in Table 1 may not be indicative of each breed's average. For example, Overman *(10)*, in later studies of the milk of Brown Swiss cows in 39 herds, reported the milk composition to be: 3.97% B.F.; 9.16% S.N.F.; 3.52% protein; 4.90% lactose; and 0.74% ash.

Data from Combs, Reaves, and Graf *(3)* showed that the S.N.F. of Guernseys is 9.18%, which is considerably lower than Overman's figure.

In the Holstein-Friesian breed many investigators *(2, 3, 7, 14, 21)* have reported the average S.N.F. per cent to be between 8.46 and 8.60; whereas, others *(2, 3, 7, 14, 17)* have found the average S.N.F. per cent for Jersey cows to be from 9.32 to 9.53. Robertson's *(15)* figures for composition of milk of Ayrshire cows in Scotland were similar to Overman's values.

Recently, Rolleri *et al. (16)* have studied the differences between the five dairy breeds in the components of protein. They reported that the milk from Holstein cows has less total alpha- and beta-, but more gamma-casein, than the milk of other breeds of dairy cattle. When the proportions of the casein were considered, Holstein milk had less beta- but more gamma-casein, whereas the Ayrshire milk had more beta-casein than the other breeds.

The evidence from breed data certainly is indicative of a genetic difference in composition of the milk of dairy cattle.

Sire differences. Variation among sires in the average composition of their daughters' milk for S.N.F. has been reported by several workers *(8, 9, 14, 21)*. The interpretation of such data needs to be made with caution when such data extend over a long period of time, since environmental factors such as age, climate, and feed may be responsible for a sizable portion of the sire differences. However, recent information from Holland and Great Britain *(13, 15)* indicates that daughters of sires used in A.I. centers, and sires whose daughters are tested in special progeny test stations, exhibit differences in protein and S.N.F. content of their milk.

Individual differences. The evidence for heritable differences in milk composition between individual cows has received only scant consideration from research workers. The bulk of the evidence stems from studies of identical twins in Sweden and New Zealand *(4, 5, 6, 22)*. The heritability figures derived from research with identical twins are given (Table 2). Such figures include not only the additive gene effects but also dominance and epistatic effects. Robertson believes that these figures are biased upwards and include an environmental biats.

The use of dam-daughter relationships to estimate heritability (in the narrow sense, because

[1] Paper No. 682, Department of Genetics.

TABLE 2

Heritability estimates (twin data)

Source	Component of milk	Heritability
	(%)	
Sweden *(5)*	Fat	0.87
	Protein	0.88
	Lactose	0.62
New Zealand *(4)*	Fat	0.95
	Casein	0.94

all the dominance effects and most of the epistatic effects will be missing) of milk composition has been reported by Robertson *(15)*. The data used in his study were age-corrected, single-lactation records of 300 dam-daughter pairs of the Ayrshire breed. The estimates of heritability are given (Table 3). Also in this

TABLE 3

Heritability estimates (dam-daughter data)

Source	Component of milk	Heritability
	(%)	
Scotland *(15)*	Fat	0.32
	Protein	0.48
	Casein	0.58
	Lactose	0.36
	Ash	0.50
	S.N.F.	0.53
Netherlands *(13)*	Protein	
	Lactose	0.5 to 0.7
	S.N.F.	

table are presented some values computed by Politiek *(13)* in an analysis of Friesian data in the Netherlands. The two reports show that heritabilities of individual components of milk are moderately high.

There have been many studies of the association between some of the constituents of milk *(12, 18)*. However, in this report only the data presented by Robertson *(15)* are given (in the right-hand portion of Table 4). The lower portion of Table 4 shows the genetic correlations between constituents, also reported by Robertson. The genetic correlations between casein and both fat and lactose were positive and significant. The genetic correlation measures the association between two characters, because the same genes have effects on both characters. The phenotypic correlation contains this genetic component and also a contribution from environmental factors affecting both traits. The genetic correlation is useful in predicting change in one character while selecting for another. Because of the small number of pairs involved in this study, the standard error of the genetic correlation is large.

Summary

Evidence has been presented which indicates that part of the variation in composition of milk is genetic and that selection for S.N.F. or its basic constituents should be effective in changing the composition of milk of our future dairy-cow population. However, additional data are needed in order to obtain more precise estimates of the genetic parameters, i.e., heritabilities and genetic correlations.

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TABLE 4

Phenotypic and genetic correlations (within herds)

	Yield	Fat	S.N.F.	Crude protein	Casein	Lactose	Ash
Yield		−0.14	−0.18	−0.26	−0.20	0.08	−0.05
Fat	−0.01		0.40	0.42	0.43	0.11	−0.03
S.N.F.	−0.02	0.46		0.81	0.87	0.50	0.12
Crude protein	0.22	0.48	0.94		0.93	−0.01	0.10
Casein	0.05	0.56	0.82	0.96		0.17	0.00
Lactose	−0.16	0.37	0.67	0.41	0.41		−0.28
Ash	−0.10	−0.42	−0.42	−0.10	−0.24	−0.86	

Phenotypic correlations

Genetic correlations

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PROPOSED PLAN FOR COOPERATIVE STUDY OF THE INHERITANCE OF MILK COMPOSITION

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Experiments concerning the inheritance of economic factors not previously studied extensively involve essentially the same pattern of procedure; namely, environmental factors which affect characters, adjustments which should be made, and the genetic parameters which can be estimated.

I. General considerations

A. *Environmental factors affecting milk composition:*

1. Age at calving
2. Stage and length of lactation
3. Month of calving
4. Breed
5. Calving interval, days dry, gestation period
6. Other factors: geographic and managemental effects, disease and other unusual circumstances.

B. *Adjustments to be made.* Using methods of least squares, one can measure and adjust for the effects found to be significant. These adjusted data then can be used to estimate the following genetic parameters:

Repeatability indicates the reliability of a single record as an indication of breeding value. If it is of sufficient magnitude, cows could be sampled for a single lactation or for periods during a lactation.

Heritability represents the relative magnitude of the genetic variability and, depending on its magnitude, indicates the selecting procedures which should be followed to achieve maximum genetic progress.

Genetic and phenotypic correlations of the milk components with each other and with other production characters to provide information required to establish optimum schemes for simultaneous selection of several traits.

II. Recommended procedures

A. Collecting much data for several years will be necessary to estimate adequately the desired statistics. Herds could be selected which would give a high frequency of daughter-dam pairs or which would maintain daughters of AB sires.

It is recommended that cattle in various geographic areas, and in various herd levels within these areas, be sampled routinely by the DHIA Supervisor. At least two locations each should be selected, in the East, South, Midwest, and West, to sample cows in various production levels.

Sampling 3,000 animals in each breed for from 3 to 5 yr. should result in sufficient records to estimate repeatabilities and heritabilities. These figures must be arbitrary, based on statistics which vary among herds and probably are, at best, only incompletely known.

B. Methods of testing will depend on the available testing procedures and on the importance of the following components:

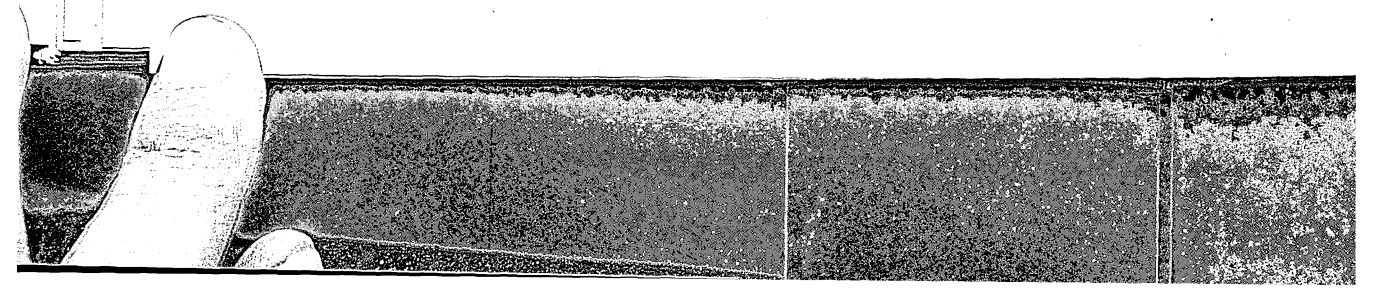
Solids-not-fat is most important economically and can be measured accurately in the field by the DHIA Supervisor, using the small Watson Lactometer. Therefore, maximum effort should be placed on solids-not-fat.

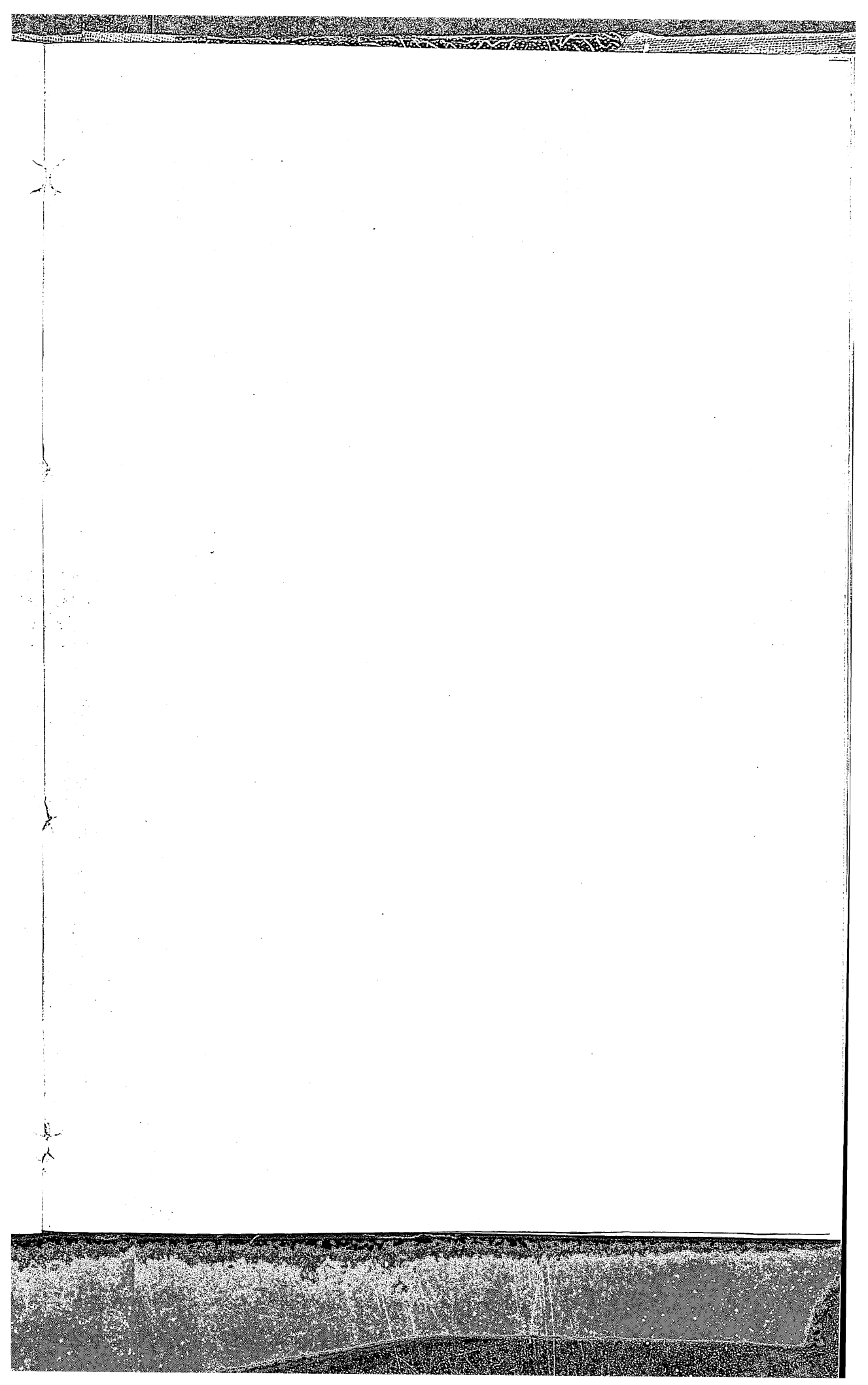
Protein, total nitrogen, protein components, etc. are important to know, both economically and physiologically. These cannot be measured accurately in the field, and they involve tedious and expensive laboratory procedures. Some of these detailed data should be obtained on large numbers of cows, but they will be expensive and difficult to obtain.

Lactose and minerals are relatively unimportant economically, but have sufficient physiological importance to warrant laboratory consideration.

C. Testing equipment and methods should be investigated continuously, in an attempt to estimate milk components more accurately and economically.

The suggested procedures indicate that solids-not-fat should be measured extensively, that protein measurements should be made (but will be limited at present) on as many animals as possible, and that limited consideration should be given to lactose and ash.





OUR INDUSTRY TODAY

THYROPROTEIN FOR DAIRY CATTLE

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Thyroprotein is a hormone-containing product which produces an increase in milk production when fed to dairy cattle. The question of whether the increase in milk production is sustained, along with other questions of feeding the product, is discussed in the following presentation. The product has been available commercially since about 1945; its sale has varied considerably from time to time and from locality to locality and reached a minimum about 1954. Recent sales effort has increased the use of the product in some areas.

The product is manufactured by the iodination of casein under certain specified conditions. The active agent in the product is the hormone, thyroxine, the same hormone that is secreted by the thyroid gland. The thyroprotein is usually mixed with some feedstuff and sold as a supplement to be fed in addition to the usual ration.

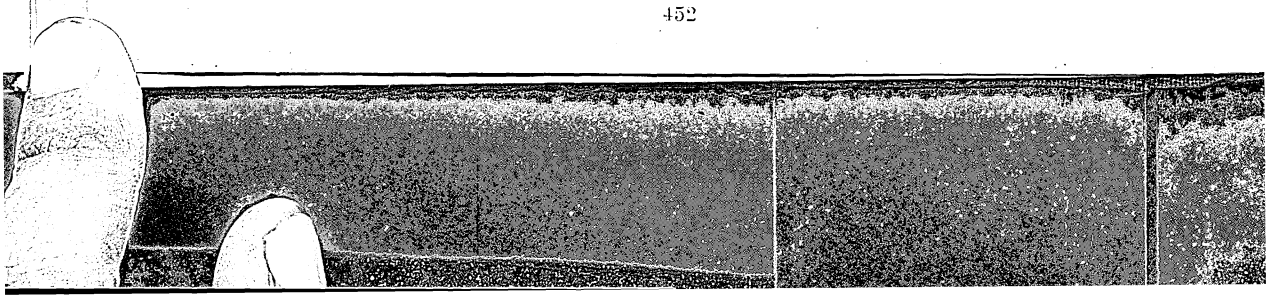
The most recent technical review of the various aspects of feeding thyroprotein to dairy cattle was published by the National Research Council *(5)*. The review of the English and American work was concerned largely with the effects of feeding thyroprotein over a greater part of the lactation period. It was pointed out that the feeding of thyroprotein results in an average temporary increase in milk production of from 15 to 20% above control animals, but that the total milk production for the lactation was not increased when the same level of energy intake was maintained. The following statement in the summary of the review is given, "The available data suggest no definite economic advantage of feeding thyroprotein to dairy cows under most farm conditions. It appears more desirable for the dairyman who raises his own herd replacements to refrain from using thyroprotein, especially if the calf mortality observations are confirmed. It may, however, be advantageous to use thyroprotein in well-fed commercial herds in which maximum production per cow is emphasized, and in herds during periods of short milk supply where this is permitted by law and where other regulations do not prohibit its use. It probably should not be fed for periods longer than 90 to 120 days and not during the first 50 days of lactation or in the terminal part of lactation."

The question of interpretation of response following thyroprotein feeding needs some discussion, especially in relation to energy intake of control and thyroprotein-fed animals. Two procedures have usually been followed in both short- and long-term experiments. *(1)* The control group is fed according to requirements, whereas the thyroprotein group is fed either the requirements plus the nutrients in 3 to 5 lb. of concentrate or at a certain given per cent above requirements. As milk production increases, owing to thyroprotein stimulation, grain is proportionately increased. *(2)* Both groups are fed according to requirements for maintenance and milk production or a certain per cent above requirements.

It was found in the early results reported from Beltsville and discussed in the Technical Review *(5)* that if No. 1 was followed, in which thyroprotein was fed 50 days post-partum for the remainder of the lactation period, milk production for the thyroprotein-fed animals exceeded the control animals for the lactation period. This effect is, of course, owing both to extra feed and to thyroprotein. When No. 2 was followed, milk production for the thyroprotein-fed animals was above that of the controls for a period of from 60 to 120 days, after which the level fell below that of the controls, so that there was little difference in production for the lactation period, regardless of whether both groups were fed at requirements or at 25% above requirements. This measured the effect of thyroprotein per se. Therefore, it was concluded from the long-term experiments that unless the dairy farmer is interested in producing extra milk for a certain period for the market it would appear very doubtful that it would pay to feed thyroprotein. He probably could obtain about the same production for the lactation period by feeding extra grain without thyroprotein.

Thus, in measuring effects, No. 1 is due to extra feed and thyroprotein, whereas No. 2 is due to thyroprotein. It is felt that the latter procedure provides the only fair basis to assess the true value of thyroprotein. Only when a distinction is made between extra feed and thyroprotein effect can the true effect of thyroprotein feeding be assessed. This is true for both short- and long-term feeding of thyroprotein. This consideration usually has not been made clear in the popular press, in articles which discussed thyroprotein feeding, where no account of the extra feed intake has been considered and with the inference that the results are owing solely to the feeding of thyroprotein.

Although control cows usually will not consume as much feed as thyroprotein-fed cows, they will consume more than the Morrison



standard requirements, and many individual cows, if given the opportunity, will approach the amount consumed by thyroprotein-fed cows.

In unpublished data from Beltsville, five sets of identical twins were used, in which one animal of each set was fed thyroprotein at the rate of 1.2 g. per 100 lb. of body weight, beginning 50 days post-partum for the remainder of the lactation. Extra feed was offered simultaneously, equal to 125% of Morrison's maximum requirement. The other member of each set served as a control, being fed no thyroprotein but fed at 125% of requirement. The controls produced 9,734 lb. of FCM for the lactation, whereas the thyroprotein-fed animals produced 9,245 lb. Actual consumption of TDN for the entire lactation as compared to Morrison's requirement was 106% in both groups. One set of triplets was included in the experiment; one of the triplets was fed at Morrison's standard and produced 9,110 lb. of FCM; another of this triplet set was fed at 125% of Morrison's standard and produced 11,195 lb.; the thyroprotein-fed triplet was fed at 125% and produced 9,988 lb. of FCM. Actual feed consumption in comparison to Morrison's standard was 93, 111, and 109%, respectively. These results confirm the early long-term feeding experiments conducted at Beltsville and again point up the importance of feed or energy intake in the interpretation of results.

Since the reporting of the long-term thyroprotein-feeding results from Beltsville and publication of the Technical Review *(5)*, attention has been turned toward short-term feeding of thyroprotein, for periods of from 60 to 120 days. When thyroprotein is fed for short periods, questions concerning the yield of milk and economic returns have been in recent controversy as affected by, *1)* the length of the period of stimulation of milk production in relation to production for the lactation; *2)* the level of feed intake; and *3)* the method of withdrawal of thyroprotein from the ration. Results to answer some of these questions are available, although there is need for rationalization of disputed points in the light of the previous discussion.

Experiments on short-term feeding of thyroprotein to obtain information on milk production and economic return have been conducted at Beltsville *(6, 7)* and at the University of Tennessee *(3, 4)*. In the experiments at Beltsville *(6, 7)*, a total of 38 controls and 81 cows fed thyroprotein at the rate of 15 g. per day for 60 days have been used at four different locations under varying conditions. Accurate feed consumption data, including both concentrates and forage, milk production, and live weight records, were obtained. Data on the pre-experimental and post-experimental periods also were collected. A typical result is shown (Figure 1).

Again, it was shown that when thyroprotein was fed, milk production was increased. How-

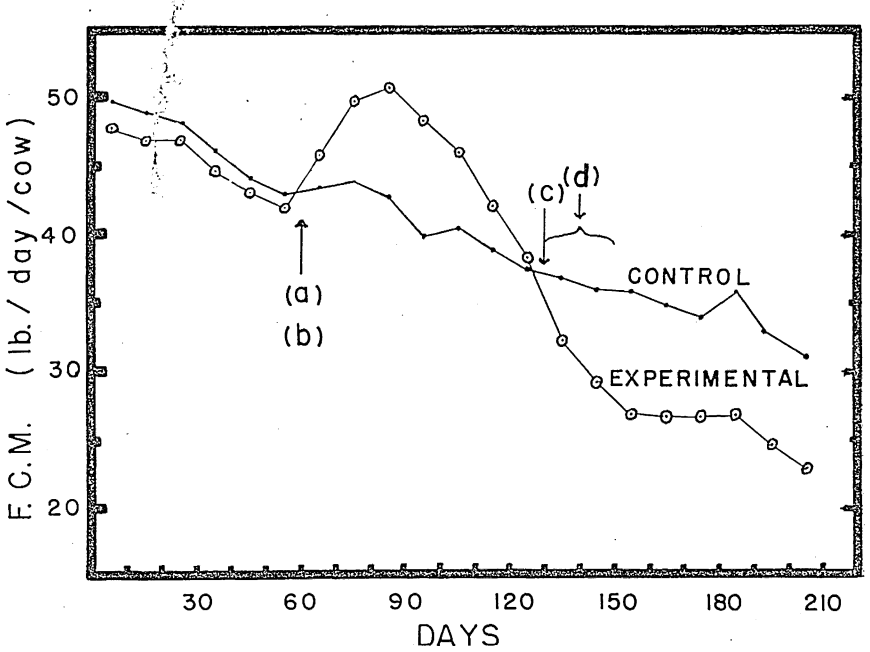


FIG. 1. FCM production of eight control and 24 experimental cows. (a) Extra grain fed to controls, (b) thyroprotein and extra grain fed to experimental cows, (c) extra grain removed from controls, (d) extra grain and thyroprotein removed from experimental cows during this period. (Data adapted from J. Dairy Sci., 40: 128. 1957.)

ever, when thyroprotein was removed from the ration, milk production decreased below the calculated expected level or the level of control animals. Calculations of monetary returns showed an advantage during the period when thyroprotein was fed. However, when the post-experimental period also was considered, much of the advantage disappeared and was dependent upon the extent of decrease in milk production which followed withdrawal of thyroprotein. Gradual withdrawal was tried and whereas milk production decreased more gradually, the final level reached was the same as when the thyroprotein was removed abruptly.

Investigators at the University of Tennessee *(3, 4)* reported that the gradual withdrawal of thyroprotein following a 100-day feeding period resulted in milk production decreasing only to the expected level of the lactation curve or to that of the controls. It would appear on the surface that the results obtained at the University of Tennessee *(3, 4)* and at Beltsville *(6, 7)* were in conflict. However a study of the data shows that the results can be rationalized in the light of the previous discussion. It is believed that the differences are due to the level of feeding of the controls in relation to the thyroprotein-fed cows during the period of thyroprotein feeding. In the Tennessee experiments, the control cows received 19.5 lb. of TDN per day, whereas the thyroprotein-fed groups received 22.2 lb. of TDN per day, or a difference of 2.7 lb. during the 100-day feeding period.

In the Beltsville short-term experiments, with one exception, the control and thyroprotein-fed groups were generally equal in their consumption of TDN, with the greatest difference being about 1.5 lb. per day. Where extra grain was added there was some concomitant decrease in hay intake which tended to equalize TDN intake. Thus, in most cases the Beltsville results actually measured the difference due to thyroprotein per se and not that due both to feed and to thyroprotein. It is believed that the results at Tennessee, in which the thyroprotein-fed animals did not decrease in yield below the controls, are not due to the gradual withdrawal of thyroprotein but to the level of energy intake. In this interpretation, there is really no point of disagreement between the results obtained at the University of Tennessee and those at Beltsville. However, it should be pointed out that the cows at the University of Tennessee were fed thyroprotein for 100 days, compared to 60-90 days at Beltsville.

In the work of Blaxter *(1)* in respect to withdrawal experiments, close reading of the text and a study of the data reveal agreement with the concept at Beltsville. He states that gradual withdrawal reduces the rate of decrease in milk production. Examination of his data where feed intake was equalized reveals that the final level of milk production was not affected, whether the withdrawal was sudden or gradual.

The effects of feeding thyroprotein on subsequent milk production and viability of calves have been in controversy. In the earlier long-term experiments conducted at Beltsville, evidence of a decrease in production levels and poor viability of calves during successive lactations was reported. No such evidence has been obtained in short-term feeding experiments. However, a word of caution should be expressed where thyroprotein is fed during the latter part of the lactation, in terms of the possible effects noted in the long-term feeding experiments. It has been noted that when thyroprotein is withdrawn from the ration, the thyroxine-secretion rate returns to normal in a period of from 3 to 4 wk. It has been stated that because thyroxine secretion returns to normal there should be no effect on the subsequent lactation or on the viability of calves born during subsequent pregnancies. Obviously, the fact that the thyroxine-secretion rate had ample time (90 days) to return to normal in the earlier long-term experiments did not prevent the effects previously noted. The effects must, therefore, be owing to some other disturbance in metabolism which was not corrected by a resumption of the normal thyroxine-secretion rate.

In this connection, it is of interest to note the results of an experiment reported in a paper of Hancock, Brumby, and Turner *(2)*, in which six identical-twin heifers were injected daily for the first 6 mo. of gestation with L-thyroxine at the rate of 0.4 mg/100 lb. of body weight. During the succeeding 3 mo. of gestation, the previously injected heifers largely caught up with their control twins in body weight and apparently regained their normal thyroxine-secretion rate. Despite the fact that both groups were treated similarly after the thyroxine injections, the heifers which had been previously injected with the thyroxine produced less milk, 3,932 to 5,051 lb. of FCM, and had shorter lactations than the control twins in their first lactation. This and other evidence reported by English workers indicates that thyroprotein feeding may cause shortened lactations.

The question of whether to feed thyroprotein for short periods is largely one of economics, considering the price of milk and the price of feed. If the farmer is establishing a base period in the market, and there is considerable advantage in the class and price of milk, it may pay him to feed thyroprotein to selected cows during the base period and sacrifice milk production later, provided the price of grain is not excessive. The feeding of extra grain without thyroprotein will usually not immediately stimulate the cows to extra milk production but will promote greater persistency. If there is no advantage to immediate stimulation for the base period, there would be no advantage on the total lactation period to feeding thyroprotein, even though the milk : grain price ratio was advantageous.

Thyroprotein should not be fed to herds when poor management exists and when the herd is underfed. Under any given situation, it should be fed only to those herds which are well-fed and well-managed. Under such a situation, the heavy feeding of grain would produce as much milk for the lactation as would feeding extra grain and thyroprotein.

Efficiency of feed utilization is increased during the short-term periods of thyroprotein feeding. However, if the post-experimental period also is considered, then there is no advantage in efficiency of feed utilization.

Some evidence indicates that thyroprotein should not be fed during the hot summer months, because of the combination of heat stress and the hyperthyroid state. Of course, the effect would be more serious in the southern than in the northern states.

Most of the evidence indicates that thyroprotein should not be fed to first-lactation animals. The greatest response has been obtained during the third and succeeding lactations. Thyroprotein should be fed only during the declining lactation phase; preferably not during the late stages of the lactation period.

The feeding of thyroprotein causes considerable loss in live weight. This effect is probably due to two factors; namely, actual loss of body fat due to the hyperthyroid state and a change in body composition and/or fill. Weight loss from the latter causes is regained readily when thyroprotein is removed from the ration; whereas, that owing to body fat loss must be compensated for by extra feed.

In conclusion, it can be stated that the feeding of thyroprotein for short periods to well-fed and well-managed cows, under special market situations, may prove to be profitable, but that the feeding of extra grain without thyroprotein will produce as much milk for the lactation as extra grain and thyroprotein. The feeding of thyroprotein by the general dairy farmer should not be advised.

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PROBLEMS IN THE ITALIAN SOFT-CHEESE INDUSTRY[1]

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Originating centuries ago in southern Italy were two well-known soft cheeses, Mozzarella and Ricotta. These fit together well in the cheese-manufacturing scheme because one cheese results from the side product of the other.

Traditionally, Ricotta, or recooked cheese, is made from the whey of Mozzarella and other "pasta-filata" types. To this whey a small amount of skimmilk is often added to improve the final texture. A joint application of acid and high heat results in a protein precipitate which is removed as cheese.

Few appreciate the world-wide importance of the "pasta-filata" principle of cheese-making. In this system the texture and grain of the cheese are achieved through a skillful stretching of curd in hot water. Provolone, Caccicavallo, and other cheeses owe their characteristics to this principle. Yet, it is a soft, nonaged cheese, the Mozzarella, which is the foundation for all pasta-filata type cheeses. Knowledge of the manufacture of Mozzarella provides a good start on knowledge of these other cheeses.

It would be interesting to speculate on the circumstances leading to the origin of Mozzarella and Ricotta, one of which was described by Varro in 37 B.C. The stretching of the curd in the former, and the flotation principle of curd in the latter, antedating that of Ivory soap, reflected certain signs of ingenuity on the part of the innovators.

Ricotta cheese in the United States. New York City is the mecca for Ricotta, especially at

[1] Presented at Research Conference at Beltsville, Maryland, October 30, 1957, held by the Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

Easter-time, but other cities are becoming important consumer centers. Ricotta is used directly in salads, in ravioli, in cheese cake, and as cream-whipped fillings for desserts. It can be consumed also just as one eats cottage cheese. Good quality Ricotta has a soft, fragile texture and a sweet, walnut flavor.

In the East, for many years cheeses from whole milk and from part skim, usually 2% fat, have become much more popular than the product from whey. The following classification is in general use:

Ricotta—cheese from whole milk
Part-skim Ricotta
Ricotone—cheese from whey
Dry Ricotta.

In areas other than the East, relatively little cheese is made from whole or part skimmilk and the term Ricotta usually denotes cheese obtained from whey.

Principles of Ricotta cheese manufacture. Whole milk is pumped into large stainless steel kettles and enough lactic acid starter is added to adjust the titratable acidity to 0.29-0.31%. It requires from about 20 to 30 min. to heat the milk to the critical temperature level for precipitation, 175-180° F. Direct steam injection is used for the initial heating.

The first sign of a precipitate is observed by the operator as fine flocculent material, on the flat surface of a spatula which is moved constantly through the heating milk. At this point the direct steam injector is removed and heating is continued slowly by jacketed steam under 5-10 p.s.i. During this period, the curd collects at the top of the kettle, with the operator making simple smooth, sweeping movements of the curd with his spatula from the surface edge to the center. After a brief quiescent period, the curd is scooped off into perforated metal containers. These containers are cooled in running water so that the cheese attains a temperature of 40° F. as rapidly as possible before shipment to market.

Salt and ice cream stabilizer also are normal additions to cheese milk intended for Ricotta. Salt highlights the flavor and perhaps contributes to a more complete coagulation. The ice cream stabilizer prevents heavy accumulation of foam, so that the cheese-maker can detect more quickly the first signs of protein flocculation.

Though starters are the most widely used means of introducing an acid to the milk, acetic acid, tartaric acid, and phosphoric acids also are employed. Along certain Mediterranean Sea areas, sea water is often used in the manufacture of a cheese similar to Ricotta. The heavy salt concentration evidently results in a salting out of the protein.

Problems of precipitation. Difficulties are common in the coagulation of proteins of whole milk and skimmilk Ricotta. An almost simultaneous precipitation of casein, lactoalbumin, and lactoglobulin is required. At times, conditions are not entirely suitable and a second precipitation must follow, to recover fully the cheese curd. The color of the whey, clear green or milky, is a good indicator of the efficiency of the initial precipitation.

In at least two periods of the year, when cows go out to pasture in the spring, and upon their return to the stables in the fall, pronounced difficulties in effecting complete precipitation are encountered. More starter and more heat are necessary. The relative proportion of proteins in the milk and shifts in salt equilibria, so critical to evaporated milk stability, must exert an influence, but in this instance the problem is stability and not instability.

If the color of the whey in the kettle after initial precipitation is milky, sufficient citric acid is added, with brief heating, to induce a secondary coagulation. The resulting cheese is coarse and rough and is blended in with the better quality first cheese.

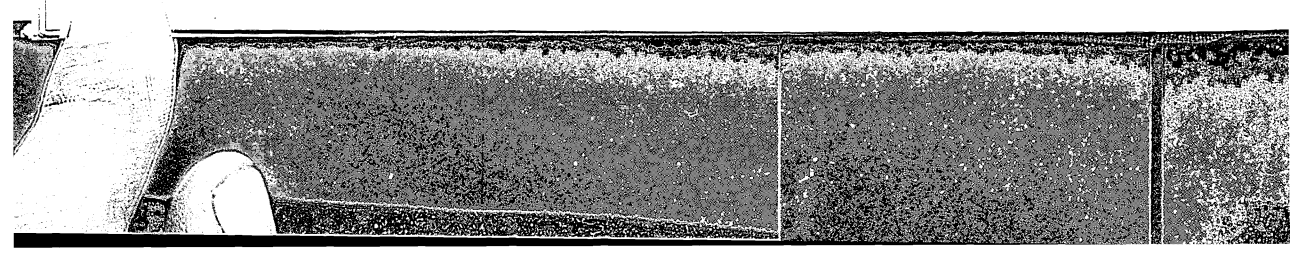
The turtle of the Ricotta cheese family. It is a curious sight in the manufacture of Ricotta cheese to see the mass of curd rise to the top of the kettle in an almost simultaneous movement. Why don't these curd particles settle to the bottom like most good precipitates? Occluded air between and in the curd particles must explain this action resulting in a density of the curd mass slightly lower than the liquid in which it exists. At best, this is a delicate equilibrium.

A related cheese exists, however, which made in the same manner and from the same fresh milk, will sink to the bottom of the kettle where it stays. Called Impastata, it is utilized by bakers because it makes a finer whipped pastry filling. It differs from true Ricotta by being drier and having a higher lactose concentration. The acidity of the milk through starter addition is the contributory cause for its sinking. Milk with an acidity adjusted to more than 0.35% is most likely to behave in this manner, perhaps because the curds become tougher, contracting and forcing enough air to upset the delicate equilibrium determining the direction of the cheese's movement.

Technological advances. Unfortunately, no real improvements in manufacturing methods have evolved for Ricotta cheese. This is attributable mostly to the philosophy of the Italian cheese industry, which has resisted most suggestions for change, fearing inferior cheese and higher expense.

Continuous centrifugation based on principles which apply to the new continuous manufacture of Baker's, Neufchatel, and cream cheese has been suggested. Such a process, though warranting study, apparently has not yet been tested by Italian cheese manufacturers.

Mozzarella cheese in the United States. Approximately 24 million lb. of soft Italian cheese are produced annually in New York State, much of it Mozzarella. Its increased popularity



parallels that of the well-known Italian specialty, pizza pie. Mozzarella cheese is consumed in its natural state, in the preparation of lasagne and in veal cutlet alla Parmagiana.

Presently, some confusion exists as to the nature of true Mozzarella cheese. When made in the East, the cheese generally is white, with a soft texture and a high moisture, milky consistency, and a bland flavor. When made in the Midwest, the cheese generally is yellowish, drier, and frequently displays a slight Provolone flavor. The latter often is referred to as Mozzarella-Provolone or Pizza cheese. Eastern whole-milk cheese contains about 50% moisture and 19% fat, whereas Midwestern cheese averages 46% moisture and 23% fat.

Methods of manufacture. Mozzarella cheese produced in the East is made from pasteurized milk to which lactic acid or enterococci starters are introduced with or without vinegar. Rennet is added, to form a curd within 30 min.

The curd cut with cheese knives is not cooked but remains in the whey for a short period of time, after which the whey is removed completely. The curd bed is cut up into small blocks, washed with cold water, and bundled into cloth bags. After storage at 40° F. for 24 hr., these bundles may be shipped under ice to small heat-processing centers in distant cities. Here they are brought out to a warm room to reactivate the acid fermentation so that the pH will drop to about 5.3. At this point the curd is stretched in hot water, molded, and brine-salted.

In the Midwest, a high acid-producing starter, *L. bulgaricus,* is added to the pasteurized milk. Rennet paste or rennet is used to form a curd. The curd cut with cheese knives is cooked to 117° F. Then the whey is removed and the curds are matted as large patties. At an acidity of about 0.35%, reached in a few hours, the patties are milled into long strips and placed in hot water, where kneading takes place. The hot plastic curd mass is shaped into various forms and salt-brined.

The Eastern manufacturers, as a hedge against competition, now make some of this cheese also; whereas, the Midwestern manufacturer does likewise with the high-moisture Mozzarella. The high-moisture product upon shipment over long distances to Eastern markets has displayed difficulty in maintaining peak quality.

Chemistry of Mozzarella cheese. Mozzarella cheese owes its characteristics mainly to the action of lactic acid on dicalcium paracaseinate. At a pH between 5.2-5.4, much of this compound becomes converted to monocalcium paracaseinate, which provides the strings and the sheen of the cheese. Above pH 5.4, generally the curd will not stretch; below pH 5.2, the curd will stretch but excessive losses in fat result and the cheese becomes too tough.

Some technical considerations. The soft Italian-cheese industry was forced into using pasteurized milk. Now, generally, it is agreed that pasteurized milk makes a better cheese. Many within the industry have considered other technical changes.

Bleaching of the yellowish color of cheese milk with benzyl peroxide has been attempted experimentally. The results are not all satisfactory, because the stretching qualities of the curd are reported to be adversely affected. Similarly, homogenization of milk leads to a whiter appearance, but again the curd-stretching properties are adversely affected.

Another technical development and generally accepted practice is in the freezing of Mozzarella curd, generally after it has been acid-ripened. Frozen in periods of surplus, the curd is heat-processed to a finished product when milk supplies are low. The product quality is unimpaired.

A processed Mozzarella cheese is being considered as a related type. It is more difficult to process than cheese of the Swiss or Cheddar type, but it is not impossible. Because of its good slicing properties and long-keeping qualities, processed Mozzarella certainly deserves some attention.

SUMMARY

The Italian-cheese industry is faced with many problems, but two of immediate urgency are concerned with poor sanitary quality and a lack of state and national standards of identity.

Both Ricotta and Mozzarella cheese often are heavily contaminated with mold, yeast, and coliforms. This is particularly significant with Ricotta, because supposedly it leaves the hot kettle in an almost sterilized condition. Yet, when Ricotta cheese is packaged in plastic consumer cups, dramatic evidence frequently is displayed of poor sanitary quality through exploding caps and obnoxious flavors.

Obviously, the water which comes in contact with the cheese during slow cooling might be a source of the high counts. Designs for quick-cooling air-tunnels, replacing the water, are still on the drawing-boards and may remain there indefinitely because of estimated high cost of construction. Not to be overlooked is the fact that a lack of good cleansing and sterilizing techniques in these cheese plants may be a more important contributory factor.

In the packaging of Mozzarella cheese, mold growth may spoil the product because of the high initial count and because this cheese has no natural mold inhibitors such as propionic acid. Vacuum packaging has helped to reduce the incidence of mold in packaged cheese, but with sliced Mozzarella a high vacuum of 29 in. must be maintained for effective control.

The decision of the Federal Food and Drug Administration not to promulgate standards of identity for Ricotta and Mozzarella cheese at this time has been received with some misgivings. Standards are urgently needed for the elimination of the chaos apparent in certain segments of this industry. Manufacturers would do well

to sit down together and work out on a nation-wide scale the general basis for satisfactory standards of identity.

In the present confused state of the market, much cheese labeled and sold as Mozzarella bears little or no relationship to this cheese, whether of Eastern or Midwestern origin. Resembling poor Muenster, or green Cheddar, the entry of such cheese creates a very disturbing influence on the Italian soft-cheese industry and on many consumers.

The Italian soft-cheese industry is growing at a rapid rate. To keep it healthy, new ideas are of paramount importance. Now that many cheese manufacturers are becoming receptive to technological advances, a wonderful opportunity exists for progress.

NEW DEVELOPMENTS IN THE PROPAGATION OF STARTERS[1]

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Use of More than One Culture for Making Cheese Starter

For the past couple of years, there has been an increasing number of successful cheesemakers who have regularly used from two to four lactic cultures for developing their daily needs of bulk starter. This is in direct contrast to the general practice reported in foreign literature, where selected single-strain lactics have been in use for a number of years. Why, then, are many in our cheese industry using the more complex multiple-type, mixed-strain cultures, even increasing the complexity of the mixture by using from two to four cultures together in their bulk starter tanks?

Basically, a pertinent factor is lack of facilities, equipment, and utensils conceded to be necessary for assuring adequate protection and control of single-strain cultures.

Another factor is the long hours required for completing daily cheese-making operations in many of our factories, plus the necessity of refilling vats during many days of flush production. Clean-up periods and chances to air our factories are cut to a minimum, especially at certain times of the year.

With the general type of equipment, facilities, and practices in this country, mixing of two or more multiple-type cultures has aided many cheesemakers in achieving:

(1) More uniform results in acid development and other desired characteristics of bulk starter prepared daily.

(2) Apparent leveling out of some of the effects of the inhibitory properties of milk. It is, of course, a well-known fact that individual cultures may vary in their sensitivity to inhibitory properties frequently present in milk supplies, and the broader mixture of lactics helps minimize some of the wider swings in starter activity.

From observations made on this practice of mixing two or more cultures, maximum success can be attained when certain conditions are met:

(1) Cultures included within a mixture for starter must be compatible and proven to work well together. It must not be assumed that just any lactic culture will work well with another, because this is definitely not the case.

(2) Cultures used for the mixture should be propagated independently as mother cultures or intermediates and mixed only in the starter tank. If a group of cultures are mixed and propagated as a blend, strain dominance will commonly occur and the mixture of lactics will revert to fewer strains. The benefits of having a wide variety of compatible lactics and associates or citric acid fermenters can be lost rather quickly, sometimes within a propagation or two by cheesemakers.

(3) Protection of all cultures against infection is important. Actual cases of failure have been diagnosed where just one culture out of a good working combination or blend was found to be infected with bacteriophage.

(4) Carrying cultures too long in cheese factories may change the strains of bacteria sufficiently to remove the full benefits of the original mixture. Because of this, some plant operators have adopted the practice of using fresh packages of commercially prepared seed culture each day for making mother culture. This is frequently referred to as intermediate and is made in sufficient quantity for setting up the larger bulk-cheese starter. Mother cultures are not propagated in the usual manner of transferring from flask to flask, but directly from pure seed available in freeze dry form.

A More Specific Form of Mixing Cultures

So far, we have dealt with the mixing of multiple-type, mixed-strain cultures, which is about the broadest type of mixing that we can have. However, foreign literature reports some mixing of compatible single-strain lactics on an alternating basis. It is not unusual for pairs of lactics to be used each day, with enough

[1] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by the Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

pairs available so that different cultures can be used each day for a week or more without duplication of the lactic strains employed throughout this period.

Currently, some tests are being run on the mixing of selected single-strain lactics, combined with a compatible associate or citric acid fermenting organism. If successful, such a combination will offer a means of much greater control than the broader mixtures of more complex blends. Although it is not exactly the same idea being carried out in foreign countries, it closely approaches the same specific characteristics. In addition, there is the general feeling in this country that the citric acid fermenting bacteria are important for bolstering the hardiness of cheese cultures and for aiding in desired Cheddar cheese flavor. There is reasonably good evidence to justify these feelings on the part of cheesemakers in this country. Development of volatile acids by citric acid fermenting bacteria, combined with lactics, is well known. From the standpoint of activity, we are pleased to present data by F. J. Babel[2] which show clearly the beneficial effects on the activity of a lactic combined with a compatible associate.

Culture, 1% inoculation	Acidity after incubation at 30° C. for: 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.
Streptococcus lactis alone	0.19	0.19	0.23	0.36	0.64
Citric acid fermenter alone	0.19	0.19	0.19	0.19	0.19
Combined culture	0.20	0.21	0.35	0.66	0.82

Standardization of Medium for Cultures and Starters

The problems of assuring a suitable medium are very important.

For sustained good performance of lactic cultures, there is evidence that it is difficult to surpass high-quality skim or whole milk, which has been heated with care and within moderation. However, due to the unpredictable variations which may occur in the growth-supporting properties of natural skim or whole milk, pretested nonfat dry milk solids is being used by many cheese-factory operators. The term pretested must be emphasized, because it obviously needs a more specific definition, or meaning.

In our laboratory, we have experienced wide variations in the qualities of nonfat dry milk solids, presumably pretested for starter activity. It has not been uncommon to observe much wider variations than we desired in the characteristics of cultures propagated in different lots of reconstituted skim. This includes activity, flavor production, and body development.

To give us the confidence we desire, in adopting any given lot of powder for extended propagations of cultures, we want to know that:

(1) The powder selected will support good growth and activity of a *number* of different lactic cultures for at least a week and preferably longer. It is, of course, important to us to prove the merits of any powder for a representative group of cultures, because the reactions of one or two cultures may be quite different from others.

(2) We also inspect all cultures for flavor and body development during the trial test period.

Judging from the results observed, we feel it is in order to suggest a number of important factors which should be determined in pretesting powders for cultures and starters.

(1) Use of a greater number of cultures in testing.

(2) Observing results carefully for a greater number of propagations. A gradual decline in desired qualities of culture may take place.

(3) Testing carefully each new lot of powder against a previously proven lot for activity, flavor production, and body development. This supplies a good base for evaluation.

In England, special culture medium is, or soon will be, offered for preparing cultures and starter for cheesemaking. It is essentially a low-calcium milk solids medium, claimed to be very closely controlled and standardized for reliable culture behavior and protection against phage development.

Protective Equipment, Procedures, and Facilities

Effective control and performance of cheese cultures and starter involve protection against phage infection. We share the opinions of many investigators in referring to bacteriophage as the "Number One" enemy and control problem in the successful development and performance of cultures and starters. Control measures which are being employed in foreign countries and to a growing extent in this country in some degree, include:

(1) Provision of separate facilities for culture and starter preparation, well-isolated from general manufacturing rooms and equipped with independent filtered air inlets or air-conditioning systems.

(2) Rotation of different cultures that have unrelated phage sensitivity patterns.

(3) Use of protective equipment, including:

(a) Water-sealed, air-filtered starter cans with protected inoculating spouts. Their construction is similar to those employed for many years in New Zealand and Australia. They are available from at least one manufacturer in this country.

Briefly, the basic working principles and protective features of this equipment are: The lid, agitator, shaft,

[2] Purdue University, Lafayette, Indiana.

and inoculating spout are all water-sealed to prevent any breathing through these areas. As milk in the starter tank is heated, the expanded air escapes through a small opening at the top provided for this purpose. However, at the end of the heating period, and before cooling is started, a filtering device is attached to the small opening. Therefore, during cooling only filtered air is drawn back into the bulk starter tank. When ready for inoculation, a steam ring is placed around the covered inoculating spout and the water-sealed cover is removed through a jet of steam. The steam is directed upward and across the opening of the exposed inoculating spout to protect the opening against infection from the air. Culture is introduced into the starter tank through the steam-protected inoculating spout and the water-sealed cover is put back into place.

(b) Air-tight equipment which avoids any exchange of air during heating and cooling. Inoculations are made through serum-type rubber stoppers, with the equivalent of hypodermic needles. An integral part of this system is the use of polyethylene bottles, sealed with serum-type rubber stoppers. Specially designed inoculating needles are used for transferring from bottle to bottle as well as bulk tanks. Such equipment is shown in photographs (Figures 1-4) which were taken in a large, modern cheese factory in England. The basic principle involved in the use of this equipment is to avoid any exposure of cultures or bulk starter to air infection. Figure 1 shows 10- and 40-gal. starter cans.

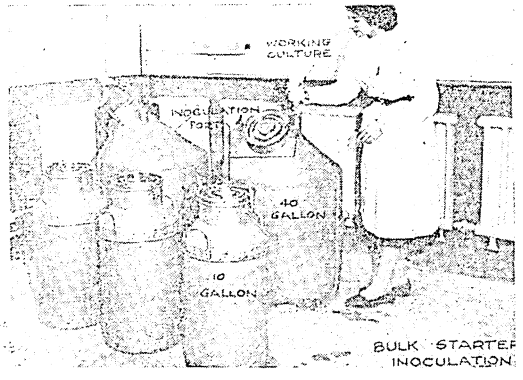


FIG. 1.

Each container is made air-tight by the use of a specially designed tight-fitting closure. A clamping device is used to force a rubber seal against the neck of the cans and a serum-type rubber stopper is used to plug the small opening provided for inoculation.

Figure 2 shows the large tank used for heating and cooling the starter cans of milk, which are completely submerged under water during heating and cooling.

Figure 3 shows polyethylene bottles being used for mother cultures. These range from

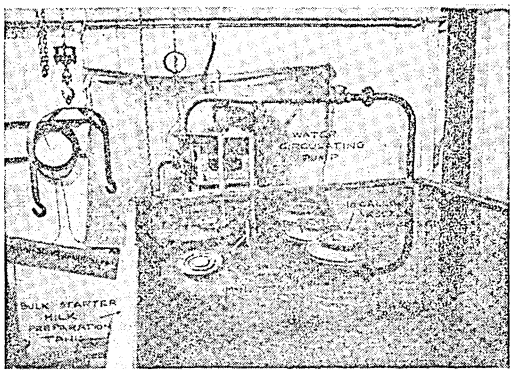


FIG. 2.

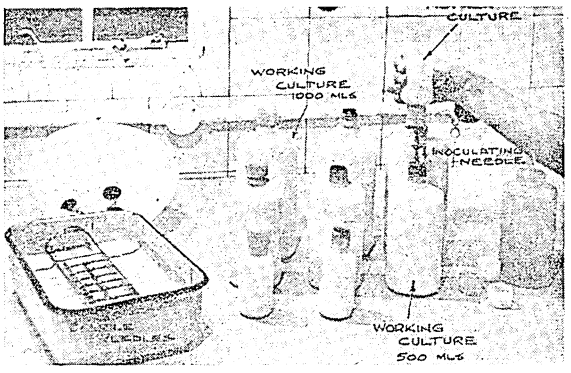


FIG. 3.

about 4 oz. for basic seed up to 1-liter (approximately 1 qt.) size for providing the inoculum for bulk cans. The bottles are plugged with serum-type rubber stoppers, and transferring from one to another is accomplished by the use of a specially designed double-needled device, as illustrated.

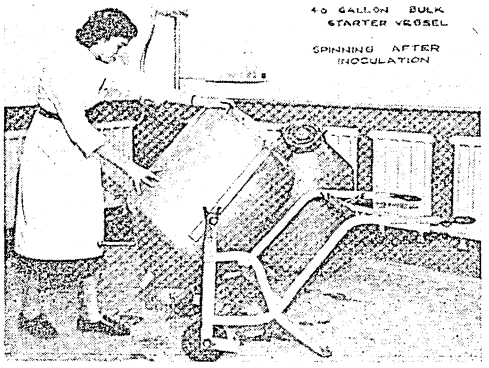


FIG. 4.

The polyethylene bottles are flexible enough to permit discharging the culture when they are squeezed.

Figure 4 shows one of the 40-gal. cans being revolved in a special cradle for mixing the inoculum. The cradle device with wheels also provides a means of transporting the starter cans to a room maintained at 70° F. for incubation.

The figures illustrate the measures which have been taken, principally for protection against phage infection and consequent failures and cheesemaking losses. Whether we agree or not, substantial evidence has been offered for the need of such control measures and the benefits resulting therefrom. Outstanding improvements have been reported following their use in cheesemaking.

If phage-resistant media prove successful for use in this country, phage-control measures may take a different form. However, we need proven results under representative commercial conditions, plus availability of such media.

OUR ASSOCIATION

STUDENT INTERNATIONAL CONTEST IN JUDGING DAIRY PRODUCTS

C. J. Babcock

Director, Poultry Division, Foreign Agricultural Service, USDA, Washington, D. C.

The 23rd Collegiate Student International Contest in Judging Dairy Products, sponsored by the Dairy Industries Supply Association and the American Dairy Science Association, was held in San Francisco, October 21, 1957. The contest was under the supervision of C. J. Babcock, Director of the Dairy and Poultry Division, Foreign Agricultural Service, U. S. Department of Agriculture, assisted by D. R. Strobel, Deputy Director of the Division. The official judges were: *Cheese:* H. L. Wilson, Kraft Foods Company, Chicago, Illinois; *Butter:* N. E. Fabricius, Flambeau Valley Farms, Ladysmith, Wisconsin; *Ice Cream:* J. H. Erb, The Borden Company, Columbus, Ohio, and *Milk:* D. A. Pettee, Creamery Package Manufacturing Company, Boston, Massachusetts. Their associate judges were Neal Angevine, Charles Weinreich, Vern Wilt, and Dan Roahen, from the Dairy Industries Supply Association judging committee.

The efficiency of the judges was checked by Professors E. D. McGlasson and G. H. Watrous for milk; L. J. Manus and W. S. Rosenberger for butter; M. P. Dean and E. W. Custer for cheese, and G. Ilg and W. S. Slatter for ice cream.

The team from the University of Connecticut was first in judging all products and received the all-products bowl and a Fellowship for $1,800 from the Dairy Industries Supply Association. The State College of Washington was in second place and received a Fellowship for $1,650 from the same Association. Cups, wrist watches, and medals for team proficiency in judging single products and for individual accomplishments were scattered widely, as shown by the following listings of the first ten individuals and teams for each of the products and for all products.

CHEESE

Individuals

Rank		*Score*
1.	Robert West, Fresno State College	30.00
2.	Gene Christman, Ohio State University	30.34
3.	Floyd Bodyfelt, Oregon State College	30.55
4.	Ronald Valinoti, University of California	31.01
5.	Edward Geishecker, University of Connecticut	31.26
6.	Harold Suelter, Kansas State College	31.42
7.	Barney Wallis, Mississippi State College	31.67
8.	Tom Townsend, Kansas State College	31.98
9.	LeRoy Iverson, University of Minnesota	32.51
10.	Lawrence Wills, University of Minnesota	33.26

Teams

Rank		*Score*
1.	Kansas State College	97.71
2.	University of California	98.58
3.	Mississippi State College	102.36
4.	University of Connecticut	102.75
5.	University of Minnesota	104.61
6.	Oregon State College	104.71
7.	State College of Washington	106.35
8.	Ohio State University	107.35
9.	Utah State University	107.94
10.	University of Wyoming	110.33

BUTTER

Individuals

Rank		*Score*
1.	David Duda, Iowa State College	6.00
2.	Larry Weatherly, University of Missouri	7.50
3.	W. Richard DuKelow, University of Minnesota	9.50
4.	Ernest Fleuette, University of Connecticut	10.00
5.	Edward Geishecker, University of Connecticut	10.51
6.	Robert Ingram, Oregon State College	11.00
7.	Larry Groves, Iowa State College	11.50
T 8.	Larry May, University of Missouri	12.00
T 8.	Gene Christman, Ohio State University	12.00
10.	Keith Yeager, University of Wyoming	12.50

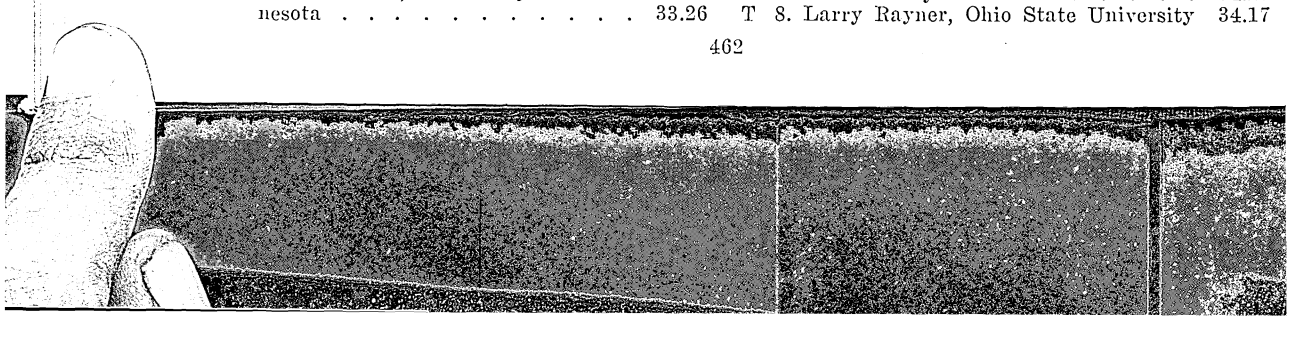
Teams

Rank		*Score*
1.	University of Connecticut	33.50
2.	University of Missouri	33.50
3.	Iowa State College	34.00
4.	University of Minnesota	41.00
5.	University of Wyoming	44.17
6.	Ohio State University	45.17
7.	Oklahoma State University	45.42
8.	Oregon State College	45.50
9.	University of Wisconsin	46.27
10.	State College of Washington	46.34

ICE CREAM

Individuals

Rank		*Score*
1.	Raymond Bay, State College of Washington	29.00
2.	Charles Neikirk, Ohio State University	29.67
3.	Larry Weatherly, University of Missouri	30.00
4.	Arley Olson, State College of Washington	30.84
5.	Richard Fallert, University of Missouri	31.67
6.	Barney Wallis, Mississippi State College	32.00
7.	David Duda, Iowa State College	32.67
T 8.	Patrick McComas, Oklahoma State University	34.17
T 8.	Larry Rayner, Ohio State University	34.17



T 10.	Jerry Marcello, The Pennsylvania State University	34.50
T 10.	Peter Schoeck, University of Connecticut	34.50

TEAMS

Rank		*Score*
1.	Ohio State University	99.01
2.	University of Missouri	100.67
3.	State College of Washington	100.85
4.	University of Connecticut	112.51
5.	Kansas State College	114.17
6.	The Pennsylvania State University	116.00
7.	Mississippi State College	116.01
8.	Iowa State College	116.68
9.	University of Minnesota	119.76
10.	University of Nebraska	122.59

MILK

INDIVIDUALS

Rank		*Score*
1.	Tom Townsend, Kansas State College	8.75
2.	Maynard Harr, University of Nebraska	9.00
3.	Richard Drake, University of Wyoming	9.33
4.	Ernest Fleuette, University of Connecticut	9.42
5.	Harvey Smith, California Polytechnic College	11.25
6.	Patrick McComas, Oklahoma State University	12.50
T 7.	Raymond Bay, State College of Washington	12.92
T 7.	Donald Raistakka, State College of Washington	12.92
9.	Larry Groves, Iowa State College	13.00
10.	Charles McGowne, California Polytechnic College	13.25

TEAMS

Rank		*Score*
1.	University of Nebraska	37.67
2.	State College of Washington	40.09
3.	California Polytechnic College	42.33
4.	University of California	42.75
5.	Kansas State College	44.25
6.	University of Wisconsin	46.60
7.	University of Connecticut	46.67
8.	University of Missouri	49.51
9.	Iowa State College	49.84
10.	Oklahoma State University	54.25

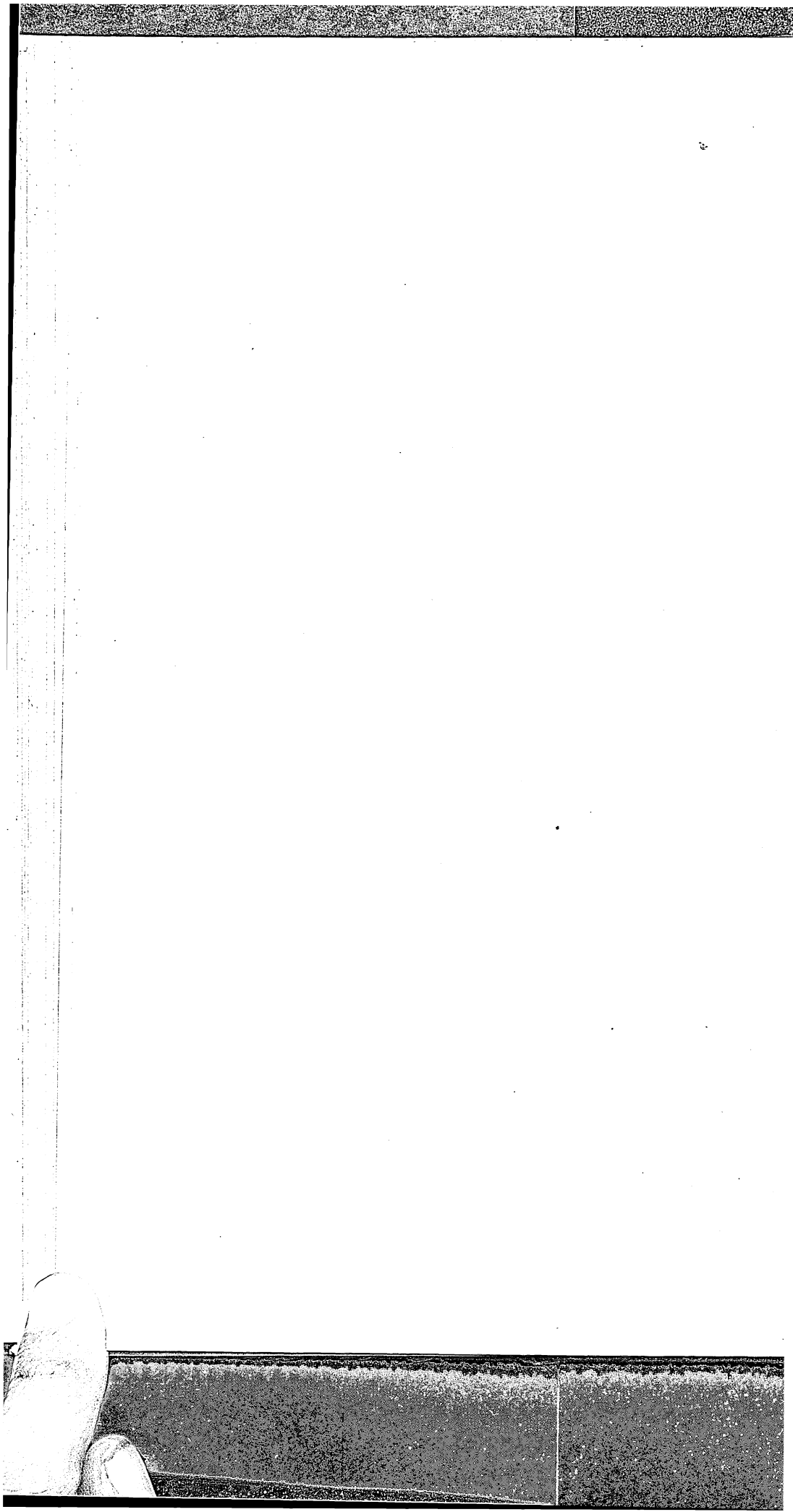
ALL PRODUCTS

INDIVIDUALS

Rank		*Score*
1.	Ernest Fleuette, University of Connecticut	47
2.	Tom Townsend, Kansas State College	52
3.	Raymond Bay, State College of Washington	57
4.	Larry Weatherly, University of Missouri	59
5.	Donald Raistakka, State College of Washington	63
6.	Gene Christman, Ohio State University	65
7.	Lloyd Vierro, University of California	67
8.	Harold Suelter, Kansas State College	73
9.	David Duda, Iowa State College	74
10.	Peter Schoek, University of Connecticut	75

TEAMS

Rank		*Score*
1.	University of Connecticut	16
2.	State College of Washington	22
3.	Kansas State College	26
4.	Ohio State University	27
5.	University of Missouri	28
6.	University of Minnesota	35
7.	University of California	36
T 8.	University of Wisconsin	37
T 8.	Iowa State College	37
10.	University of Nebraska	39



EFFECT OF CALCIUM CHLORIDE AND HEAT ON SOLUTIONS OF MIXTURES OF β-LACTOGLOBULIN AND CASEIN

E. S. DELLA MONICA, J. H. CUSTER, AND C. A. ZITTLE
Eastern Regional Research Laboratory,[1] Philadelphia, Pennsylvania

SUMMARY

The effect of heat and calcium chloride concentration on the precipitation in the ultracentrifuge of casein and β-lactoglobulin has been studied. With β-lactoglobulin alone (1% solution) at pH 6.5 with 2 m*M* calcium chloride per liter, the protein is totally precipitated after heating at 90° for 30 min. and ultracentrifuging (127,000 × G for 45 min.). With β-lactoglobulin (1%) and casein (2%) together under the same conditions, no precipitate is obtained until the calcium chloride concentration exceeds 4 m*M* per liter. Electrophoretic examination of the supernatant solution, when 50% of the total protein is precipitated (8 m*M* calcium chloride per liter), together with the insolubility of the precipitate in versene, suggested that the β-lactoglobulin is in the sediment. This conclusion was confirmed by the determination of the phosphorus to measure the casein distribution. Similar treatment of an unheated mixture showed that all of the β-lactoglobulin was in the supernatant solution. Also determined was the amount of calcium bound to the precipitated proteins, both alone and mixed. The total results support the assumption that the proteins in the mixture studied react independently and not as a complex.

In the course of studying the effect of heat and calcium chloride concentration on the precipitation of β-lactoglobulin *(9)* and casein *(10)*, it was observed that in a mixture of β-lactoglobulin and casein, the β-lactoglobulin was not precipitated with a concentration of calcium chloride that would precipitate it alone. Since several reports *(3, 6)* have indicated that β-lactoglobulin and α-casein form a complex when the two are heated together, the precipitation of the mixture of β-lactoglobulin and casein by calcium chloride was studied quantitatively, to see whether there was evidence for complex formation. The solubility studies were also supplemented with electrophoretic studies of the soluble and precipitated fractions. The effect of heat on a β-lactoglobulin-casein mixture containing no calcium was also studied electrophoretically.

MATERIALS AND METHODS

Proteins were purified and solutions prepared as previously described for β-lactoglobulin *(9)* and whole casein *(10)*. α-Casein was prepared by the urea purification method *(4)*.

The protein solutions were heated at 90° C. as described previously *(9)*. Ten milliliters of solution were heated for 30 min., then cooled to 30° C. in a constant-temperature water bath. This controlled cooling was necessary because of the reversal of the calcium caseinate precipitation *(10)*. The solutions were

Received for publication August 26, 1957.

[1] A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

then centrifuged for 45 min. at 127,000 × G in the Model L Spinco ultracentrifuge.[2] The supernatant solution (soluble fraction) was removed by decantation, and calcium and protein concentrations were determined as described previously *(10)*. Unheated solutions were allowed to stand at room temperature for 2 hr., for maximum aggregation by the calcium, before centrifuging as described above. The total protein concentration in mixtures of β-lactoglobulin and casein was determined from the light absorption at 280 mμ. Fortunately, the absorption factors for these two proteins are about the same (0.93 and 0.85, respectively) and a weighted average factor of 0.88 was used. Thus, even if all of one of the proteins was precipitated, the maximum error in the protein determination would be about 4%. The validity of this factor for the mixture was confirmed by absorption measurements with known mixtures.

RESULTS

In Figure 1 is shown the precipitation in the ultracentrifuge of β-lactoglobulin, casein, and a mixture of the two after heating at 90° C. for 30 min. in the presence of 0.5 to 18.0 m*M* of calcium chloride per liter. It is evident that β-lactoglobulin alone is totally precipitated at a low concentration of calcium

[2] Mention of products does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

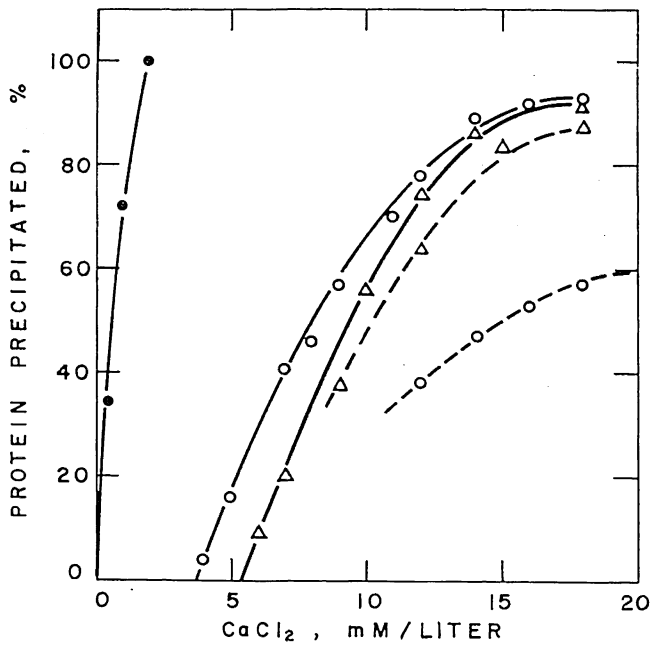


FIG. 1. Precipitation in the ultracentrifuge of β-lactoglobulin, casein, and a mixture of the two with heat (90° C. for 30 min.) and calcium chloride at pH 6.5. Also shown is the precipitation of unheated casein and a casein-β-lactoglobulin mixture with calcium chloride. Heated (solid lines): ●————●, β-Lactoglobulin (1%); Δ————Δ, Casein (2%); o————o, Casein (2%)-β-Lactoglobulin (1%) mixture. Unheated: same symbols, dashed lines.

chloride (2 m*M*/liter). In the mixture of β-lactoglobulin (1.0%) and casein (2.0%), however, a precipitate is not obtained until the concentration of calcium chloride is close to that required to precipitate casein alone.

The possibility that β-lactoglobulin might predominate in the precipitate from heated mixtures of casein and β-lactoglobulin at low concentrations of calcium chloride was suggested by the fact that in this region, the total protein precipitation with the mixture was greater than with the casein alone. To determine the composition of each fraction (precipitate and supernatant solution), a mixture of the proteins was prepared containing 8 m*M* of calcium chloride per liter and heated as before. The two fractions were separated in the ultracentrifuge. The precipitate could not be dissolved with calcium-binding versene solution. This is consistent with the idea, though not conclusive proof, that β-lactoglobulin predominates in this fraction, since calcium caseinate dissolves in the presence of versene *(10)*. The clear supernatant solution over the ultracentrifuge sediment was subjected to electrophoresis, after equilibrating with two liters of veronal buffer, pH 8.4 μ 0.1. The pattern obtained after electrophoresis for 3 hr. was that of whole casein, 27% β and 73% α, with mobilities of 3.0 and 5.8×10^{-5} cm^2/volt/sec, respectively. The concentration peaks were symmetrical and there was no evidence of β-lactoglobulin in this pattern. Thus, all of the β-lactoglobulin appears to be in the precipitate, and since about 50% of the total protein was precipitated, β-lactoglobulin composed 66% of the precipitated fraction.

The above evidence for the precipitation of β-lactoglobulin at lower calcium concentration than the casein has been substantiated by determining the distribution of phosphorus in these solutions. The phosphorus serves as a convenient tracer for locating the casein. The results of these analyses are given (Table 1). The composition of the starting mixture is 33.3% β-lactoglobulin and 66.7% casein. A solution containing 5 m*M* calcium chloride per liter, when heated and

TABLE 1

Protein composition, estimated from phosphorus content, of ultracentrifuge sediments from heated solutions containing β-lactoglobulin (1%) and casein (2%), with various concentration of calcium chloride

	Protein distribution		Phosphorus distribution[a]		Casein in sediment		
$CaCl_2$ conc.	Supernatant soln.	Sediment	Supernatant soln.	Sediment[b]	Total casein[c]	Total protein in sediment[d]	β-lactoglobulin in sediment[e]
(m*M*/l)	(%)						
5.0	78.6	21.4	99.9	0.1	0.1	0.3	64
6.0	60.0	40.0	96.2	3.8	3.8	6.3	113
7.0	57.1	42.9	90.0	10.0	10.0	15.6	109
8.0	48.5	51.5	80.2	19.8	19.8	25.6	115
9.0	44.8	55.2	64.2	35.8	35.8	43.3	94

[a] Total phosphorus in 10.0 ml. of starting solution was 1.54 mg.
[b] Estimated by difference.
[c] Measured by per cent phosphorus in sediment.
[d] Per cent in Column 6 times 66.7, divided by Column 3 times 100.
[e] Estimated from per cent of total protein in sediment (Column 3), less casein in sediment (Column 6 times 66.7), divided by β-lactoglobulin in 100 parts of mixture (33.3) times 100.

ultracentrifuged, has 21.4% of the total protein in the sediment. Phosphorus analyses indicate that only a negligible amount of the precipitate, namely 0.3%, is casein. With 6 m*M* calcium chloride per liter, 40% of the total protein is precipitated, and of this precipitate, 6.3% is casein and 93.7% is β-lactoglobulin. This accounts for 100% (113% by calculation) of the β-lactoglobulin. Deviations from 100% (115 to 94%) are the result of analytical errors magnified by subtracting numbers.

Although complex formation has not been suggested for unheated mixtures of β-lactoglobulin and casein, the effect of calcium chloride in precipitating such mixtures was studied to serve as a control. These results are also shown (Figure 1).

In the unheated solutions, calcium chloride precipitation of the mixture is considerably less than that of casein alone. Since unheated β-lactoglobulin itself is not precipitated with calcium chloride *(11)*, the amount of the mixture precipitated was calculated on the basis of casein alone (divide by two-thirds, since casein is two-thirds of the mixture), assuming that the binding of calcium by the β-lactoglobulin does not change the calcium concentration materially. When this is done, the amount of the casein precipitated from the mixture is approximately equivalent to that precipitated from the casein alone. An unheated mixture of 1.0% β-lactoglobulin and 2.0% casein containing 18 m*M* of calcium chloride per liter was ultracentrifuged and the two fractions were subjected to electrophoresis as described. The precipitate contained β- and α-caseins (mobilities of 3.2 and 5.9, respectively) and no β-lactoglobulin. The supernatant solution contained β-casein, β-lactoglobulin, and α-casein (mobilities of 3.2, 5.1, and 5.8, respectively).

Mixtures of β-lactoglobulin and α-casein containing calcium chloride were also studied electrophoretically for evidence of complex formation. The solution contained 0.25% β-lactoglobulin and 0.75% α-casein with 2.5 m*M* of calcium chloride per liter, and was dialyzed against two liters of cacodylic acid buffer, pH 6.5, μ 0.1, containing the same concentration of calcium chloride. When unheated, there was no evidence for complex formation, and the respective mobilities were 2.4 and 5.2 [mobilities are decreased some by the presence of calcium ions *(8)*].

When a similar solution was heated for 30 min. at 90° C. and prepared for electrophoresis in the same way, the results showed that the bulk of the protein moved with a mobility of 5.1 and that a trace moved with a mobility of about 3.1. A similar heated experiment with no calcium chloride showed the bulk of the protein moving with a mobility of 5.9 and a trace at 3.3. Thus, with or without calcium chloride these experiments appear to provide evidence for complex formation. However, the explanation appears to lie in the observation of Briggs and Hull *(1)*, that the mobility of β-lactoglobulin increases when heated under certain conditions.

A solution of 0.5% β-lactoglobulin alone at pH 7.0, heated at 90° C. for 30 min., was prepared for electrophoresis as described. Electrophoresis showed that the bulk of the protein had a mobility of 3.9, not much more than that of native

β-lactoglobulin. When heated in the presence of 0.1 *M* phosphate at the same pH, however, more than a third of the protein was transformed to a product with a mobility of 6.0. Moreover, if the heating was at 80° C., as Briggs and Hull *(1)* had observed, transformation to the higher mobility was almost complete. Thus, environmental factors make an important contribution to this transformation of β-lactoglobulin, and the presence of casein might make an added contribution.

The precipitation data shown (Figure 1) were supplemented with determinations of the calcium bound to the precipitates. The results of these determinations are shown (Figure 2) for 2% casein and for the mixture of β-lactoglobu-

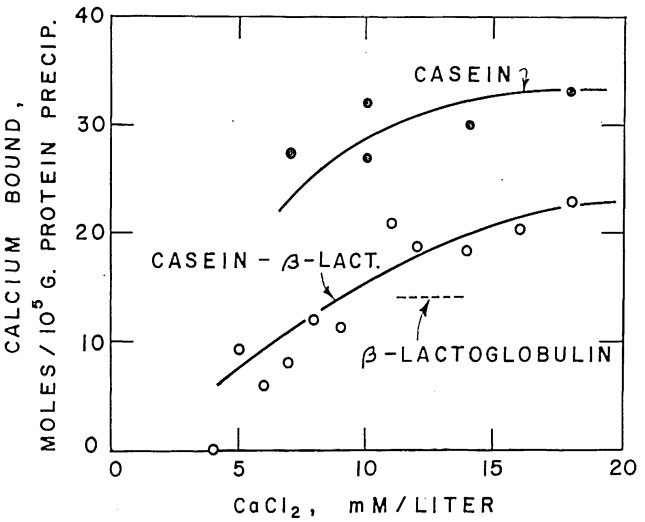


FIG. 2. Binding of calcium to casein (2%) and to a casein (2%)-β-lactoglobulin (1%) mixture at pH 6.5, precipitated with calcium chloride by heating for 30 min. at 90° C. The dashed line indicates the maximum amount of calcium bound to β-lactoglobulin at this pH.

lin and casein, heated for 30 min. at 90° C. The results favor the assumption that the β-lactoglobulin predominates in the precipitates obtained with the lowest concentrations of calcium chloride. For example, at 9 m*M* of calcium chloride per liter, where approximately 50% of both casein and the mixture are precipitated, it is apparent that considerably less calcium is bound to the precipitate of the mixture than is bound to that of the casein alone. However, if all the β-lactoglobulin is assumed to be in the precipitate, its composition would be 1 β-lactoglobulin to 0.5 casein and, using the respective binding values of 14 and 30 moles per 10^5 g., the calculated binding in the mixed precipitate is 18. This value is close to the measured binding in the mixture at this calcium concentration. Binding values for the sediments from the unheated mixtures were of the same magnitude as for casein alone. This is to be expected since, without heat, casein is the only component of the mixture that is aggregated by the calcium, and there is no evidence for complex formation between the casein and the β-lactoglobulin.

Some precipitation experiments were also done with mixtures of α-casein and β-lactoglobulin. As one might anticipate *(11)*, since the net negative charge of

α-casein is greater than the average net charge for whole casein *(5)*, about 10% more calcium is bound to α-casein than to whole casein. Contrary to expectations, however, at a given calcium concentration more α-casein than whole casein is precipitated. The calcium binding with mixtures of α-casein and β-lactoglobulin led to the same conclusions as the experiments with whole casein and β-lactoglobulin; namely, that the proteins did not interact to form a complex.

DISCUSSION

Electrophoretic experiments alone do not provide conclusive evidence as to whether β-lactoglobulin and α-casein form a complex when heated, because the heating of β-lactoglobulin solutions increases their electrophoretic mobility *(1)* until it is close to the mobility of α-casein *(6)*. Since temperature, salts, and probably other factors determine the extent to which the β-lactoglobulin is converted to a form of higher mobility when heated, the conditions for obtaining a satisfactory control are difficult to define. For these reasons, it was hoped that a study of the precipitation of the mixture of β-lactoglobulin and casein with calcium chloride could provide evidence for or against complex formation.

The precipitation studies (Figure 1) show that in the mixture β-lactoglobulin is not precipitated by a concentration of calcium chloride that will precipitate it when heated alone. Possible explanations appeared to be that the casein, by sharing the calcium chloride (the calcium-protein association constant of each is approximately the same, but casein binds more total calcium per unit weight than β-lactoglobulin), reduced the concentration available to the β-lactoglobulin below the concentration that would cause the precipitation. Or, the reduced precipitation of β-lactoglobulin might be owing to formation of a complex with the casein. One point of interest was that the mixture began to precipitate at concentrations of calcium chloride that did not precipitate casein alone. If the properties of the casein in the mixture had not changed very much, one might expect that precipitation would take more calcium, because it would now be distributed over 3 instead of 2 g. protein. This suggested that β-lactoglobulin might be the predominant component in the precipitates obtained with low calcium concentrations. The electrophoretic examination of the supernatant solution (50% of the total protein) obtained with 8 m*M* of calcium chloride per liter, together with the insolubility of the precipitate in versene, suggested that all of the β-lactoglobulin was in the sediment and composed 66% of it. This conclusion was confirmed by the determination of the phosphorus distribution. When the concentration of calcium chloride is 6 m*M* per liter, 40% of the total protein is precipitated and all the β-lactoglobulin is found in this precipitate. The total evidence indicates that β-lactoglobulin is not complexed with casein but that, instead, it precipitates independently of the casein as soon as the concentration of its share of the calcium is adequate. This interpretation of the composition of the precipitates is also supported by the relatively low amounts of bound calcium.

Experimental studies on skimmilk have not provided decisive evidence for coaggregation or complex formation. Ramsdell and Whittier *(7)* had observed

a constant difference between unheated and heated skimmilk in the amount of acid-precipitable nitrogen remaining in the supernatant as the proteins were progressively removed by centrifugation. They concluded from this that the whey proteins were not in the ultracentrifuge sediment and had, therefore, not coaggregated with the casein. Edmondson and Tarassuk *(2)* have pointed out that the nonacid-precipitable fraction will be constant, whether the heat-denatured proteins are removed in part by centrifugation or by acid precipitation alone; hence, that such experiments can not show whether coaggregation takes place. The experiments of Edmondson and Tarassuk with heated ultracentrifuge-whey suggested that the heated whey proteins are readily sedimented in the ultracentrifuge. The ability of versene to dissolve the precipitates obtained at various times of sedimentation of heated skimmilk should be a means of deciding whether whey proteins are coaggregated with the casein (versene will dissolve calcium caseinate, but not β-lactoglobulin). The phosphorus content of the sediments also might decide whether the phosphorus-free whey proteins were present.

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EFFECT OF PHOSPHOLIPIDS AND UNSAPONIFIABLE MATTER ON OXIDATIVE STABILITY OF MILK FAT[1]

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SUMMARY

Stability of milk fat samples prepared by different methods was measured by determining the length of the induction period at 80 ± 0.5° C. Fat extracted from cream or buttermilk with a 2:3 ether-pentane mixture had greater stability than churned fat. This was attributed to synergism between phospholipids and other antioxidants or synergists extracted from membrane material and plasma, and was confirmed by the stabilizing effects of adding to churned fat different levels of phospholipids and/or unsaponifiable matter. Churned fat contained no phospholipids. Unsaponifiable matter (free of acids) alone, added to churned and extracted fats, increased fat stability. Among the known constituents of the unsaponifiable matter, beta-carotene acted as a prooxidant, and cholesterol had no significant effect. Alpha-tocopherol was the only unsaponifiable constituent tested that acted synergistically with phospholipids in stabilizing churned fat. However, differences in the amounts of tocopherol could not entirely account for the differences in stability among different extracted fats. Oxidative deterioration was detected organoleptically in churned fat before the end of the induction period and at peroxide values less than 1.00.

Oxidative deterioration of lipids in dairy products, accompanied by development of oxidized flavors, continues to be an important problem. It is generally accepted that oxidation of the unsaturated fatty acids of the milk triglycerides and/or phospholipids is always involved *(7, 18, 19)*. However, our understanding of the role of nonglyceride constituents of milk fat in relation to susceptibility to oxidation is confused. The course of oxidation and production of off-flavors may vary with the type of dairy product, because of differences in composition, physical and chemical properties, and storage conditions *(13)*. Most attention has been given oxidized flavors of milk, but the mechanism is not yet clearly understood *(23, 27)* and may involve nonglyceride constituents of both milk fat and plasma *(8, 17)*.

Although certain phospholipids are readily oxidized, crude commercial phospholipid mixtures of vegetable origin have long been used as antioxidants for edible fats and oils *(19, 21)*. Such preparations are more effective in vegetable oils than in lard and other rendered animal fats. This behavior may be explained by the synergistic effect of phospholipids with the natural antioxidants, such as tocopherols, that are already present in vegetable oils. El-Rafey *et al. (2)* reported that the oxidative stability of butteroil or dry milk fat was increased when there was an increase in the phospholipid material transferred

Received for publication October 7, 1957.

[1] This study was supported in part by funds from the California Dairy Industry Advisory Board.

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from the non-oil phase of butter and an increase in the concentration of reducing substances in the butteroil. They suggested that these substances acted synergistically with the phospholipids to prolong the induction period of the butteroil.

Various mechanisms have been postulated to explain the action of synergists in reinforcing the effect of antioxidants. Calkins and Mattill *(1)* and Golumbic *(6)* proposed that the function of a synergist was the continual regeneration of the antioxidant at the expense of the synergist. Evans *et al. (3)* considered that the main mechanism may be based on the inactivation of traces of catalytically active metals such as copper or iron. Privett and Quackenbush *(25)* suggested that synergists suppress the antioxidant's catalysis of peroxide decomposition. They further postulated that phosphoric acid and its derivatives not only perform the same function as other synergists but are also capable of reacting directly with peroxides to prevent their accumulation. As pointed out by Lemon *et al. (20)*, more than one mechanism may be involved in enhancing the activity of antioxidants.

A number of investigators have studied the stability of different fractions of milk fat. Krukovsky and Sharp *(16)* prepared crystalline fractions by stepwise cooling of milk fat from 30 to −4° C. The fraction separated at the lowest temperature had the least oxidized flavor at the end of two years of storage at 4-5° C. *(15)*. Musset *et al. (22)* obtained liquid and solid fractions by hydraulic expression of milk fat crystallized at 0, 10, and 21° C. Each fraction was stored at room temperature for periods up to 4 mo., then was reconstituted in fresh skimmilk. The liquid fractions deteriorated in flavor much more rapidly than did the solid fractions, but the defect was considered to be nonoxidative *(14)*. Henderson and Jack *(9)* obtained less complex fractions by fractionating milk fat from pentane at low temperatures. Concentrated in the −53° C. filtrate were the constituents that give milk fat its unique flavor, unsaponifiable components, and glycerides with higher proportions of unsaturated and short-chain saturated fatty acids. Preliminary experiments in this laboratory revealed that this fraction was less stable to oxidative deterioration than the original fat or precipitates separated at −7, −13, −23, and −53° C. When held at 80° C. for 96 hr., the peroxide value of the −53° C. filtrate was 17.85, compared to 1.61, 1.10, 12.02, 11.17, and 14.86 for the original fat and precipitates separated at −7, −13, −23, and −53° C., respectively. Furthermore, unsaponifiable matter added to the original fat lowered its stability. These results prompted the present investigation on the relation of phospholipids and nonglyceride components to the oxidative stability of milk fat. Fat samples of different composition were prepared to determine the role of different lipid constituents on stability.

EXPERIMENTAL PROCEDURE

Materials. "Churned" fat was obtained by churning cream, melting the butter granules, and filtering the clear fat at 50° C. through Whatman No. 1 filter paper. "Extracted" fat was prepared by shaking 250-g. lots of cream with 200 ml. of methanol for 30 sec. After 5 min., 400 ml. of a 2:3 mixture of ethyl ether and pentane was added and the mixture again shaken 30 sec. When the

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aqueous phase had separated, it was drained off and the ether-pentane solution dried overnight with anhydrous sodium sulfate. "Buttermilk" fat, obtained from buttermilk left from the preparation of churned fat, was extracted as above, except that 200 g. of buttermilk was extracted with 200 ml. of methanol and 275 ml. of 2:3 ether-pentane mixture. Solvents were removed by distillation at reduced pressure.

Milk phospholipids were separated from fresh commercial spray-dried buttermilk (4.77% lipids, 5.80% moisture) by butanol extraction and precipitated with acetone. Sixty g. of powder was dispersed in 120 ml. of water with a Waring blender. The mixture was extracted in turn with 350-, 100-, 100-, and 100-ml. lots of *n*-butanol. Approximately 300 mg. of crude phospholipids (3.57% P, 1.64% N) was recovered by precipitation at 4° C. with five volumes of acetone. Infrared absorption peaks characteristic of cephalin, lecithin, and sphingomyelin *(5)* were found in fractions obtained by chromatography of the phospholipid sample. A positive anthrone test for sugar *(26)* suggested the possibility that cerebrosides were also present. Therefore, phospholipids prepared as above were assumed to be representative of those associated with milk fat. Vegetable phospholipids[4] to be used for comparison were twice precipitated from pentane with acetone.

Crude unsaponifiable matter was obtained from 100-g. lots of churned fat by the procedure used to determine unsaponifiable matter in fat *(4)*. Unsaponifiable matter essentially free of acidic material was prepared by passing crude unsaponifiable matter in wet ethyl ether through an ion exchange column of Amberlite[5] IRA400, previously activated with 0.1 *N* NaOH. Beta-carotene,[4] cholesterol,[6] and alpha-tocopherol[6] were employed without further purification.

Methods. Phospholipids, unsaponifiable matter, total cholesterol, carotenoids, and tocopherol were determined as described elsewhere *(4)*.

Stability tests were carried out at 80 ± 0.5° C. in a constant-level water bath shielded from direct light. The test tubes containing 4.0 g. of sample were removed at 24-hr. or shorter intervals, the contents mixed by gentle swirling, and one-drop aliquots (approximately 15 mg.) transferred to tared test tubes with long droppers. Each tube was rapidly reweighed to ±0.1 mg. and the peroxide value of each sample determined by the Hills and Thiel *(11)* ferric thiocyanate method. Stability is expressed as induction period, i.e., the number of hours before rapid oxidation begins as indicated by a sudden increase in the rate of peroxide formation. Figure 1 illustrates the method. With most samples, peroxide values were below 0.05 at the start of the test and did not exceed 1.00 before the induction period ended. The induction periods of eight churned and extracted fats were determined with duplicate 4.0-g. samples. The values for seven varied within ±2% of their respective means, whereas the variation for the eighth was less than ±5%. Because of this reproducibility, induction periods in the later phases of the study were generally obtained from single samples. How-

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Rohm and Haas Co., Philadelphia, Pa.
[6] Eastman Kodak Co., Rochester, N. Y.

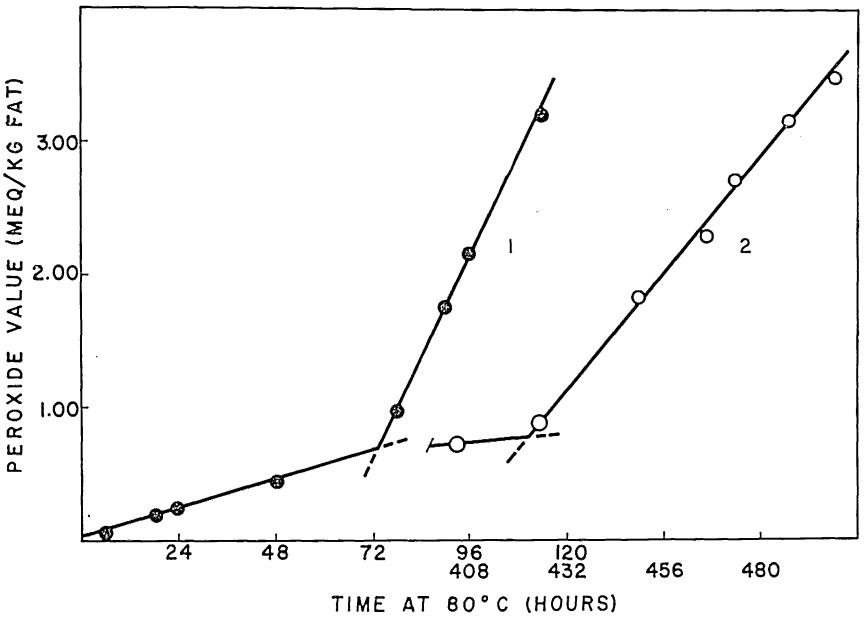


FIG. 1. Oxidation of milk fats of different composition: (1) churned fat—induction period, 75 hr.; (2) extracted fat—induction period, 425 hr.

ever, each experiment reported was repeated at least once, usually with different fats.

Each material to be tested was dissolved in a suitable solvent (usually pentane purified by passing through silica gel) and an aliquot of the solution added to the substrate. Control samples were treated with solvent only. The solvent was removed under reduced pressure in a Rinco rotating evaporator.[7]

RESULTS AND DISCUSSION

Effect of method of preparation of milk fat. Experiments 1 and 2 (Table 1) were designed to study the effect on stability of the method of preparation of milk fat. In Experiment 1, the three fats were obtained from the same lot of pasteurized cream.

Table 1 shows that the buttermilk fat and each extracted fat except C contained appreciable amounts of phospholipids, whereas the churned fats contained no phospholipids. The buttermilk fat had more unsaponifiable matter than had the other fats. Although cholesterol is the major known constituent of unsaponifiable matter, it accounted only for about half or less of the latter. There were minor differences in the amounts of cholesterol, carotenoids, and tocopherol among the fats.

The greater stability of the extracted and buttermilk fats compared to churned fat in Experiment 1 might be attributed to *(a)* higher phospholipid content, and/or *(b)* greater amounts of antioxidants or synergists extracted by the

[7] A. S. Aloe Co., San Francisco, California.

TABLE 1

Composition and stability of milk fats prepared by different methods

Sample tested	Composition					Stability
	Phospho-lipids	Unsaponi-fiable matter	Choles-terol	Carote-noids	Tocoph-erol	Induction period
	(%)			(γ/g)		(hr.)
Experiment 1						
Churned	0.01	0.33	0.20	7.3	33	75
Extracted	0.35	0.48	0.27	7.4	55	420
Buttermilk	0.75	1.53	0.39	7.9	46	570
CH + 50% EXT	(0.18)[a]	(0.41)	(0.24)	(7.4)	(44)	290
CH + 50% buttermilk	(0.38)	(0.93)	(0.30)	(7.6)	(39)	440
Experiment 2						
Churned	0.00	0.43	0.23	7.4	23	95
Extracted	0.35	0.47	0.22	7.4	31	270
EXT + 40% CH	0.26	(0.45)	(0.22)	(7.4)	(27)	230
EXT + 65% CH	0.13	(0.44)	(0.23)	(7.4)	(26)	205
Extracted A (washed cream)	0.13	0.55	0.21	7.6	....	110
Extracted B (pentane-ether)	0.22	0.44	0.21	6.8	29	230
Extracted C (pentane)	0.00	0.41	0.16	7.1	....	70

Abbreviations used in this paper: CH = churned; EXT = extracted; PLipids = phospholipids; UM = unsaponifiable matter, and FA = fatty acids.

[a] Figures in parentheses indicate calculated values.

solvents from the fat globules or from the plasma. The stability increases of 50% mixtures of churned fat and extracted and buttermilk fats were greater than the calculated average for each mixture and were not proportionate to the amounts of phospholipids or unsaponifiable matter. These data suggest the possibility of synergism between the phospholipids and some unsaponifiable constituents extracted by the solvents.

In Experiment 2, Fat A was extracted from washed cream, to determine whether the phospholipids and additional unsaponifiable matter in the extracted fats originated from the lipids associated with the fat globule membrane material or from the milk plasma or both. The plasma in the washed cream was replaced with water by diluting the cream with distilled water, reseparating, and repeating this process twice. Extracted Fats B and C were prepared by treating two portions of the original cream with ammonium hydroxide and extracting the fat with a mixture of pentane and ethyl ether, and with pentane, respectively.

Stability variations among the churned and extracted fats of Experiment 2 paralleled phospholipid content, but could not be attributed solely to this factor. When their phospholipid contents were equalized by adding 65% churned fat, the extracted fat from normal cream was more stable than that from washed cream (205 *vs.* 110 hr.). Therefore, the lower stability of Fat A extracted from washed cream may be attributed to the removal of plasma phospholipids and possibly some of the membrane lipids by the washing process. Although the washing was carried out in a stainless steel separator, there was also a possibility of contamination with metal catalyts. It seems unlikely that the minor differences determined in the amounts of known unsaponifiable matter constituents (cholesterol, carotenoids, and tocopherol) could account for the observed differences in stability.

The possibility of solvents affecting the oxidative stability was considered. Controls consisting of the churned fat to which the different solvents had been added and then removed were included. However, since in every instance the induction periods for the controls were identical with or slightly less than the sample to which the solvent had not been added, these solvent-treated controls were not included in the presentation of the data. Had there been any antioxidant effect from the solvents, the induction period would have been increased. There still remains the possibility that the extracted fat may have contained some nonlipid antioxidant material carried over by the solvents from the milk plasma. However, the presence of pentane in the solvent mixture would largely prevent water and water-soluble materials from being dissolved, just as petroleum ether removes the last traces of moisture from the ethyl ether extract in the Mojonnier fat determination. Furthermore, the ether-pentane extracts were dried with anhydrous sodium sulfate.

Table 1 indicates that both materials associated with the fat globules and others found in the milk plasma contributed to the greater stability of extracted and buttermilk fats over churned fat. The difference in induction periods of extracted Fats B and C suggests that the above constituents were extracted with a 2 : 3 mixture of ethyl ether and pentane but not with the pentane alone. Further characterization of the extracted components was not attempted.

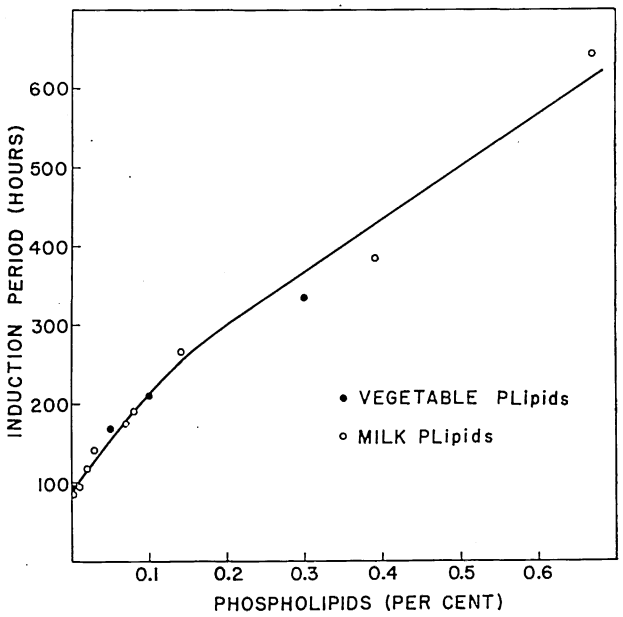


FIG. 2. Effect of milk and vegetable phospholipids on stability of churned milk fat.

Effect of added phospholipids. Experiments were carried out to investigate the effects that phospholipids added from different sources have on the stability of churned fat. Figure 2 shows that milk phospholipids and vegetable phospholipids were comparable in increasing the induction period of churned fat. The

increases in stability were approximately proportionate to the milk phospholipids added up to 0.14%. It is probable that a limiting factor at the higher levels was the amount of antioxidants in the churned fat available for synergism with phospholipids. Impure phospholipid fractions, obtained by chromatography *(4)* of buttermilk fat, also increased the stability of churned fat.

Churned fat samples became bleached toward the end of the induction period, whereas extracted fats and churned fats with added phospholipids became amber long before the end of the induction period. Absorption measurements at 440 mμ for carotenoids showed that the presence of phospholipids did not protect the carotenoids from destruction. The spectral data did not reveal any absorption maxima associated with the amber color. Evans *et al. (3)* also found that considerable darkening resulted when phospholipids containing amino-nitrogen were added to oxidizing soybean oil.

Hills and Wilkinson *(12)* reported that milk phospholipids interfered with the ferric thiocyanate test for peroxide groups in fats. They considered that the interference was analogous to the known interference by phosphoric acid in the thiocyanate test for ferric ions in aqueous solution. On the other hand, Privett and Quackenbush *(24)* and Henze and Quackenbush *(10)* suggested that phospholipids combined with fatty peroxides to form a lipid-soluble complex. They studied the antioxidant properties of wheat germ phospholipids and tomato lipids (containing phospholipids) in the oxidation of lard. The accumulation of peroxides was prevented during the induction period and both materials reduced the preformed peroxides of lard.

To determine if phospholipids interfered with peroxide determinations in the present investigation, peroxide values of churned fat held at 80° C. were determined at 24-hr. intervals in the usual manner and with benzene-methanol reagent that contained milk phospholipids equivalent to 0.1 and 0.5%, respectively, of the sample. Neither the peroxide values, which ranged from 0.05 to 3.75, nor the induction period (90 hr.) were affected. However, the same levels of milk phospholipids added to a highly oxidized churned fat, that was held at 80° C. for 2 hr., decreased the peroxide value from 9.7 to 8.2 and 6.3, respectively. These results are consistent with the observations of Privett and Quackenbush and Henze and Quackenbush that phospholipids reduce the peroxide content of oxidized fat.

Effect of unsaponifiable matter. To elucidate the over-all effect of the nonglyceride constituents in determining the stability characteristics of milk fat, unsaponifiable matter prepared from churned fat was added to churned and extracted fats obtained from the same cream. Table 2 shows that the addition of unsaponifiable matter at levels above 0.5% had a prooxidant effect and decreased the stability of churned fat. However, added unsaponifiable matter considerably increased the stability of extracted fat at all levels used. The latter result could be explained by synergism between one or more components of the unsaponifiable matter, and constituents present in extracted fat but not in churned fat—probably the phospholipids.

TABLE 2
Stability of milk fat with added unsaponifiable matter

Sample tested	Stability	
	Induction period	Change
Experiment 3	*(hr.)*	*(%)*
Churned	70	
CH + 1% UM	50	−29
CH + 4% UM	0	−100
Extracted	460	
EXT + 1% UM	670	+46
EXT + 4% UM	950	+106
Experiment 4		
Churned	110	
CH + 0.5% UM	110	0
CH + 3% UM	60	−45
Extracted	175	
EXT + 0.5 % UM	560	+220
EXT + 3% UM	800	+357

Combined effect of phospholipids and unsaponifiable matter. The postulated synergism between phospholipids and unsaponifiable matter was confirmed by adding different levels of these materials, singly and in combination, to churned fat. Experiment 5 demonstrates that phospholipids and unsaponifiable matter in combination resulted in greater stability than would be expected from their effect when added individually (see Table 3).

TABLE 3
Stability of milk fat with added milk phospholipids and unsaponifiable matter

Sample tested	Stability		
	Induction period	Change	Synergism[a]
Experiment 5	*(hr.)*	*(%)*	
Churned	100	.....	.....
CH + 0.05% PLipids	175	+ 75	.....
CH + 0.10% PLipids	205	+105	.....
CH + 0.5 UM	0	−100	.....
CH + 1.0% UM	0	−100	.....
CH + 0.05% PLipids + 0.5% UM	180	+ 80	+105
CH + 0.05% PLipids + 1.0% UM	180	+ 80	+ 75
CH + 0.10% PLipids + 0.5% UM	220	+120	+115
CH + 0.10% PLipids + 1.0% UM	200	+100	+ 95
Experiment 6			
Churned	75	.....	.....
CH + 0.05% PLipids	120	+ 60	.....
CH + 0.10% PLipids	140	+ 87	.....
CH + 0.4% UM less FA	80	+ 7	.....
CH + 0.8% UM less FA	105	+ 40	.....
CH + 0.05% PLipids + 0.4% UM less FA	150	+100	+ 33
CH + 0.05% PLipids + 0.8% UM less FA	210	+180	+ 80
CH + 0.10% PLipids + 0.4% UM less FA	170	+125	+ 31
CH + 0.10% PLipids + 0.8% UM less FA	220	+193	+ 66

[a] Synergism = % change in presence of PLipids and UM together, less % change with PLipids and UM added separately; e.g., 105 = 80 − [75 + (−100)].

The unsaponifiable matter used in the above experiments contained approximately 20% of acidic materials assumed to be fatty acids. Experiment 6 shows that the addition of unsaponifiable matter from which the fatty acids had been removed had an antioxidant effect with churned fat. Therefore, the prooxidant effect of crude unsaponifiable matter added to churned fat (Table 2) can be attributed to the acidic material present. This result is consistent with the well-known effect of fatty acids in accelerating the oxidation of fats.

Table 4 illustrates the effect of adding other known nonglyceride constituents to churned fat in the presence and absence of phospholipids. Beta-carotene acted

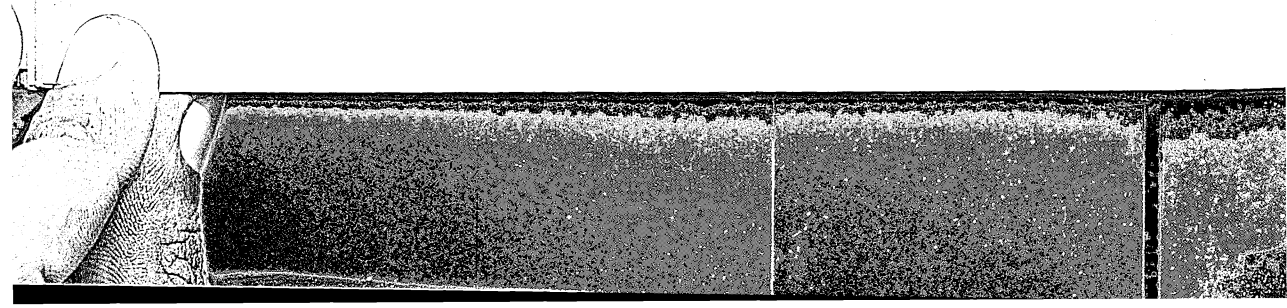
TABLE 4

Stability of milk fat with added milk phospholipids and constituents of unsaponifiable matter

Sample tested	Stability		
	Induction period	Change	Synergism
Experiment 7	*(hr.)*	*(%)*	
Churned	95	.....	.....
CH + 0.10% PLipids	265	+179	.....
CH + 10 γ/g β-carotene	85	− 11	.....
CH + 50 γ/g β-carotene	85	− 11	.....
CH + 0.10% PLipids + 10 γ/g β-carotene	240	+153	−15
CH + 0.10% PLipids + 50 γ/g β-carotene	215	+126	−42
CH + 0.5% cholesterol	95	0	.....
CH + 2.0% cholesterol	75	− 21	.....
CH + 0.10% PLipids + 0.5% cholesterol	250	+163	−16
CH + 0.10% PLipids + 2.0% cholesterol	255	+168	+10
CH + 20 γ/g α-tocopherol	120	+ 26	.....
CH + 80 γ/g α-tocopherol	140	+ 47	.....
CH + 0.10% PLipids + 20 γ/g α-tocopherol	335	+253	+48
CH + 0.10% PLipids + 80 γ/g α-tocopherol	380	+300	+74

as a prooxidant, but cholesterol produced no significant change in three of the four samples as compared to the controls. Alpha-tocopherol alone increased the induction period of churned fat in agreement with its well-known antioxidant action. It was the only constituent of unsaponifiable matter tested that also exhibited synergism with milk phospholipids. This observation is in agreement with results obtained by others *(2, 28, 29)* for vegetable phospholipids and tocopherol added to fats.

Flavor deterioration and oxidative stability. The induction period, as determined during accelerated oxidation tests, is a useful criterion of the extent to which fat resists oxidation. Oxidized odors and flavors are generally believed to arise from the decomposition of the peroxides initially formed. Unfortunately, no single chemical test has been developed to measure all the products of oxidation that contribute to the off-flavors. Traces of solvents prevented organoleptic evaluation of the extracted fat samples, but a panel of five persons detected oxidative deterioration in churned fat before the end of the induction period. For example, a churned fat (P.V. 0.05) with an induction period of 90 hr. had a very slight oxidized flavor after 16 hr. (P.V. 0.09). The off-flavor became distinct after 40 hr. (P.V. 0.16) and was tallowy after 100 hr. (P.V. 1.05). Therefore, it



appears that the peroxides developed in milk fat are relatively easily decomposed into objectionable flavor compounds. This could be attributed to labile fat peroxides, which are characteristic of the milk fat unsaturated glycerides or to some factor(s) which readily catalyze the decomposition of peroxides.

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CHROMATOGRAPHIC SEPARATION OF SOME MILK LIPIDS[1]

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SUMMARY

Fats extracted from milk and from dried buttermilk by mixtures of ethyl ether and pentane were chromatographed on silicic acid columns. Butanol was used to split the lipoprotein complexes in the buttermilk. Each fat in pentane was applied to the column and eluted with successive portions of 1, 10, 25, and 50% ethyl ether in pentane, with methanol, with acetone, and with water. Cholesterol and phospholipids were separated, but most of the tocopherols and carotenoids remained with the triglycerides. Unsaponifiable matter prepared from churned fat was applied in hexane to alumina and celite (2:1) and to magnesia and celite (1:1) columns. The material on the alumina-celite column was eluted successively with hexane, benzene, ethyl ether, and methanol. A fraction characterized as 70% cholesterol by chemical analysis and infrared spectrophotometry was recovered from the benzene eluate. The magnesia-celite column yielded 95% cholesterol in the benzene eluate. Carotenoids were separated by developing the column with hexane to obtain five clearly defined zones. These were rechromatographed to obtain homogeneity. Characteristic absorption curves and partition tests established the presence of beta-carotene, neo-beta-carotene, lutein (xanthophyll), and zeaxanthin in the eluted fractions.

Investigations of natural fats often require that complex mixtures of lipids be separated under mild conditions. Among the various techniques available for lipid fractionation, chromatographic methods have been used by several investigators and have been reviewed recently *(4, 8, 14)*. With a recent increase in interest in the atherosclerosis problem, several papers have appeared on the separation of blood lipids by chromatography *(5, 10, 15)*. *Borgstrom (2)* employed silicic acid in studies of rat lipids to separate phospholipids from neutral fat and free fatty acids. In a further study, with fats containing radioactive fatty acids *(3)*, he separated cholesterol esters from glycerides and free fatty acids. Fillerup and Mead *(4)* used silicic acid columns to separate artificial lipid mixtures and blood lipids into fractions composed mainly of sterol esters, triglycerides, fatty acids, sterols, and phospholipids.

Chromatography has been used in separating the component fatty acids *(9)* and carotenoids *(6, 13, 18, 23)* of milk fat, but the technique has been little used in separating other milk lipids. Morice *(12)* used chromatography to confirm the presence of cholesterol, xanthophyll, beta-carotene, and vitamin A in the unsaponifiable matter of milk fat. She also isolated lanosterol, and four other compounds, which were unidentified.

This work was undertaken in connection with a study of the oxidative stability of milk fat *(16)*, to explore the application of chromatography in the separation of some known components of milk fat and its unsaponifiable matter. It was hoped to develop methods for the separation of triglycerides, phospholipids,

Received for publication October 7, 1957.

[1] This study was supported in part by funds from the California Dairy Industry Advisory Board.

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cholesterol, tocopherol, and carotenoids, so that the role of each in relation to the oxidation of milk fat could be studied.

EXPERIMENTAL PROCEDURE

Materials. Materials chosen for analysis were "extracted fat" and "buttermilk fat". The latter was chosen because it offers a more concentrated source of milk phospholipids. Extracted fat was obtained from pasteurized cream by a method only slightly different from that described elsewhere *(16)*. It was prepared by shaking 200-g. lots of cream successively with 100 ml. of ammonium hydroxide, 200 ml. of methanol, and 400 ml. of a 2 : 3 mixture of ethyl ether and pentane for 30 sec., 1 min., and 30 sec., respectively, allowing the mixtures to stand for 5 min. after adding the ammonium hydroxide and 10 min. after adding the methanol. This procedure resulted in 90% recovery of fat. Buttermilk fat was prepared by shaking 60 g. of fresh commercial spray-dried buttermilk powder (4.77% lipids, 5.80% moisture) with 120 ml. of distilled water and 300 ml. of butanol in a separatory funnel. The butanol extract was decanted and the remaining material transferred into a Waring blender and extracted successively with one 100-ml. and two 50-ml. portions of butanol. The solvent was removed from the combined butanol extracts by distillation at reduced pressure and at a temperature below 60° C. The lipids were redissolved in a 1 : 1 mixture of anhydrous ethyl ether and pentane, and the solution was centrifuged at 5,000 r.p.m. for 15 min. to separate the suspended material. The ether-pentane layer was decanted and the residual solids were re-extracted with 30 ml. of chloroform. Then the combined extracts were dried overnight with sodium sulfate and the solvent removed at reduced pressure.

The unsaponifiable matter was prepared from 100-g. lots of churned fat by a procedure similar to that used to determine the unsaponifiable matter in milk fat (see Analytical Methods).

Analytical Methods. Determinations of unsaponifiable matter, carotenoids, and cholesterol were combined in one procedure. A 10.0-g. sample of fat was saponified and the unsaponifiable matter extracted, using amber glassware, by the method of Zscheile *et al. (23)* for the determination of carotenoids in milk fat. In the saponification step, 5 ml. of the methanol was replaced with 5 ml. of 5% methanolic pyrogallol to improve the subsequent recovery of carotenoids. The final ether extract of unsaponifiable matter was made up to a volume of 50 ml. The unsaponifiable matter was determined in a 25-ml. aliquot by Method Ca 6a–40 (starting from Step 7) of the A.O.C.S. *(1)*. Total carotenoids were determined in another aliquot by measuring the absorbance at 437 mμ (*vs.* peroxide-free ethyl ether as blank) and employing an extinction coefficient of 2040 in the calculations *(23)*.

Total cholesterol was determined by a modification of the method of Zlatkis *et al. (21)* for serum cholesterol. Their method was not applicable directly to milk fat, which produced a cloudy purple color with no distinct absorption maxima at 500 and 560 mμ (characteristic absorption maxima for cholesterol). When applied to the unsaponifiable matter of milk fat, a clear purple color was obtained, with absorption maxima high at 500 mμ and low at 550 mμ. However,

when this sample was read against a blank containing unsaponifiable matter and the reagents, excepting ferric chloride, the balanced curve exhibited a maximum at 560 mμ (Figure 1). Therefore, this wave length was selected for the modified

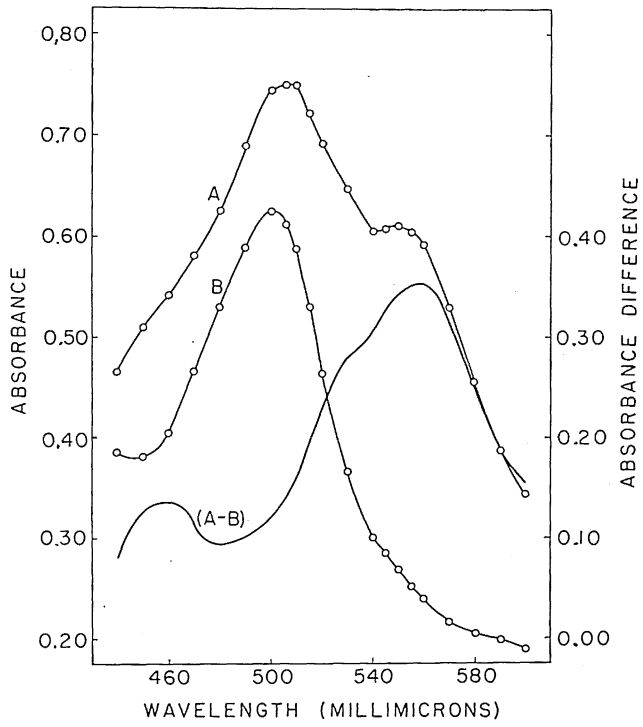


FIG. 1. Curves showing absorption maximum at 560 mμ used to determine cholesterol in unsaponifiable matter of milk fat. A—unsaponifiable matter plus acetic acid and ferric chloride in sulfuric acid, B—blank containing unsaponifiable matter plus acetic acid and sulfuric acid, and C—balanced curve obtained by subtracting adsorbances for B from A.

method employed in this investigation. Three 0.50-ml. aliquots (for blank and duplicate determinations) of ether solution of the unsaponifiable matter were pipetted into test tubes and the ether evaporated on a steam bath. Then the procedure of Zlatkis *et al.* was followed, except that the blank contained 3.0 ml. glacial acetic acid and 2.0 ml. concentrated sulfuric acid (without ferric chloride). The concentration of cholesterol was obtained from a standard curve prepared with Eastman cholesterol.[3] The recovery of cholesterol that had been added to unsaponifiable matter was 91–96%.

Tocopherol determinations were made by the method of Mahon and Chapman *(11)*, except that the acid extraction step was omitted because no synthetic dyes were present in the fats. With the standard preparations[3] of carotene and tocopherol and the Beckman DU spectrophotometer used, the correction factor for beta-carotene was 0.215 (absorbance of carotene at 448 mμ) and the "K value" for alpha-tocopherol was 0.0031.

Lipid phosphorus was determined by the method of Harris and Popat *(7)* and the factor 25.94 was used to estimate the per cent of phospholipids.

[3] Eastman Kodak Co., Rochester, New York.

D AGRICULTURAL

Chromatography. Chromatographic separation was achieved by adsorbing each lipid mixture on a column and eluting with solvents of increasing polarity. With each solvent, elution was continued until essentially complete (refractive index of the eluate remained constant or no residue was left upon evaporation).

An all-glass chromatographic apparatus was designed to permit automatic percolation of the solvent through interchangeable columns at a controlled rate (about 2–3 ml. per min.) under nitrogen pressure. Dimensions of the columns are given under the sections describing chromatography of milk fat and unsaponifiable matter. All solvents used for elution were anhydrous and redistilled. Hexane (Skellysolve B), pentane (Skellysolve A), and petroleum ether (Skellysolve F) were further purified by percolation through silica gel *(19)*. Ethyl ether was treated with sodium sulfite (125 ml. 0.25% Na_2SO_3 per liter of ether) to remove peroxides and was dried successively over calcium chloride and sodium ribbon. The adsorbents used included silicic acid (Baker's Analyzed), aluminum oxide (Baker's Analyzed), and magnesia [4] (Westvaco No. 2641). All adsorbents were activated before use by heating overnight at 120° C. With some columns, the rate of percolation was increased by diluting the absorbent with different proportions of celite.[5] The columns were packed either dry or in a slurry with solvent at reduced pressure, to aid packing. Before each run the packed columns were washed with two-column volumes of each solvent, in an order opposite to that used in the run.

RESULTS AND DISCUSSION

Chromatography of milk fat. Chromatographic separation by silicic acid was applied to the extracted and buttermilk fats. With extracted fat, a 20.0-g. sample in pentane (250 ml.) was applied to the column (100 g. silicic acid, 8 × 5.5 cm.). The eluting solvents were pentane (1,200 ml.); 1% (900 ml.), 10% (500 ml.), 25% (500 ml.), and 50% (400 ml.) ethyl ether in pentane; methanol (400 ml.); acetone (300 ml.), and water (200 ml.), in that order. Essentially the same procedure was followed in the chromatography of the buttermilk fat. Weights of the eluted fractions were calculated from the weights of residues obtained from eluate aliquots with a Mojonnier Milk Tester. Between the evaporation and weighing steps, the residues were held for 5 min. at 100° C. in the vacuum oven and cooled 7 min. in the cooling desiccator. Composition of the original fats and the fractions obtained are shown (Table 1).

The bulk of the triglycerides and carotenoids was found in the petroleum ether and pentane eluates. The cholesterol was concentrated in the fractions obtained by elution with 10 and 25% ether in pentane. The small amounts eluted with pentane were probably cholesterol esters *(4)*. The tocopherol (or reducing substances) in the buttermilk fat was separated into two main fractions of different polarity with pentane and methanol. Finally, the phospholipids in the extracted fats were eluted with methanol, acetone, and water. For the extracted fat, the phospholipids were separated into two major fractions of different polarity with methanol and water; whereas, for the buttermilk fat, the water

[4] Westvaco Chemical Division, Food Machinery and Chemical Corp., Newark, Calif.

[5] Braun-Knecht-Heimann Co., San Francisco, Calif.

TABLE 1

Chromatographic fractionation of milk fat preparations on silicic acid

Fat preparation	Fractions in order of elution	Eluting solvent	Fraction weight	Fraction constituents				
				Unsaponifiable matter	Cholesterol	Tocopherol	Carotenoids	Phospholipids
			(mg.)			*(γ)*		*(mg.)*
Extracted fat			20,000	88.4	43	586	137.0	43.0
	I	Pentane	13,995	19.3	2	.....	137.0	0.0
	II	1% Ethyl ether in pentane	2,870	14.4	0	.....	0.0	0.0
	III	10% Ethyl ether in pentane	1,980	.....	4	.....	0.2	0.0
	IV	25% Ethyl ether in pentane	645	.....	47	....	4.2	0.0
	V	50% Ethyl ether in pentane	125	.....	2	.....	0.0	0.0
	VI	Methanol	40	.....	1	.....	16.4	16.4
	VII	Acetone	5	.....	.....	.....	.....	4.2
	VIII	Water	70	.....	.....	.....	.....	15.4
Buttermilk fat			10,000	271.0	144	717	96.8	2,100.0
	I	Pentane	2,945	53.6	4	431	49.5	5.8
	II	1% Ethyl ether in pentane	1,560	8.8	0	69	17.9	0.0
	III	10% Ethyl ether in pentane	700	.....	100	.....	0.0	0.2
	IV	25% Ethyl ether in pentane	420	.....	24	.....	0.0	0.0
	V	50% Ethyl ether in pentane	55	.....	3	.....	0.0	0.3
	VI	Methanol	3,240	.....	0	244	0.0	1,208.0
	VII	Acetone	40	.....	0	.....	.....	12.5
	VIII	Water	125	.....	0	.....	.....	0.2

eluate contained a negligible amount of phospholipids. It was also noted that with each fat a red-orange band was adsorbed on the columns that could not be eluted with either pentane or ether-pentane mixtures, but only with methanol. The absorption curve of this pigment did not have the characteristic peaks of carotenoids; therefore, it probably was a carotenoid oxidation product.

The above separations are not as complete as those reported for artificial lipid mixtures and blood plasma lipids by Fillerup and Mead. Their excellent recoveries with each eluting solvent mixture, however, are based on qualitative tests and fraction weights, and any errors could, therefore, cancel out. Furthermore, their original samples contained much greater amounts of sterol esters, sterols, and phospholipids, but less triglycerides, than the fats in the present study. This factor could have reduced efficiency of separation in our work.

Other adsorbents and solvents were also investigated in the chromatographic separation of milk lipids. Alumina and magnesia caused appreciable hydrolysis of the triglycerides, as shown by increased fatty acid content of the pentane eluates. The hydrolyzing property of alumina is well known *(2, 3)*. Therefore, use of these two adsorbents for milk fat separations was discontinued.

These results show that the cholesterol and phospholipids of milk fat can be separated by adsorption on silicic acid. Most of the tocopherols and carotenoids remained with the triglyceride fractions.

Chromatography of unsaponifiable matter. Alumina and magnesia were employed for the chromatographic fractionation of the unsaponifiable matter of milk fat. In a typical run with alumina, unsaponifiable matter (0.50 g.) dissolved in hexane (200 ml.) was adsorbed on a column of a 2 : 1 mixture of alumina and celite (120 g., 16 × 3.4 cm.). Elution was carried out successively with hexane (2.4 liters), benzene (2.6 liters), ethyl ether (2.7 liters), and methanol (3.0 liters). The carotenoids were separated into four bands, each eluted with a different solvent. The hexane eluate contained approximately 50% of the total carotenoids. The cholesterol was recovered quantitatively in the benzene eluate, whose residue was 70% cholesterol. Figure 2 shows that the infrared absorption spectrogram of this material was similar to that of cholesterol from spinal cords of cattle.[3] A negligible amount of unidentified lipid material was eluted with ethyl ether and a relatively waxy fraction was eluted with methanol. This fraction consisted of approximately 50% nonashable inorganic material. Efforts to remove the contaminant from the alumina before chromatography were unsuccessful, so the use of this adsorbent was discontinued.

When magnesia was substituted for alumina in the fractionation of unsaponifiable matter, the cholesterol was eluted quantitatively with benzene, yielding a fraction containing 95% cholesterol. However, recoveries of carotenoids and tocopherols were poor under these conditions.

The carotenoids from the unsaponifiable matter were fractionated with magnesia by a procedure based on the chromatographic techniques of Strain *(18)* and Zscheile *et al. (24)*. For this purpose, churned fat was obtained from freshly drawn, raw mixed milk of Jersey and Holstein cows on pasture of mixed grasses and legumes. From this fat, unsaponifiable matter was prepared with precautions taken to reduce storage and exposure to light to a minimum. The unsaponi-

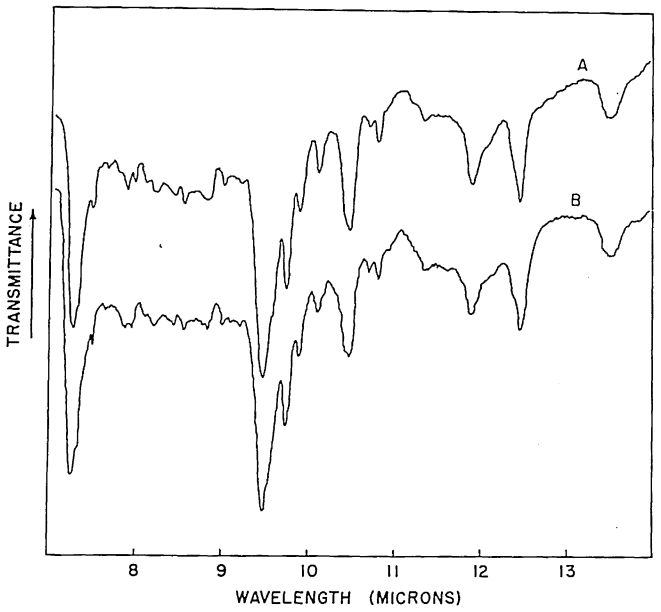


FIG. 2. Infrared absorption spectra from 7 to 14 μ of A—cholesterol from spinal cords of cattle; B—milk cholesterol; 20 mg/ml in carbon disulfide; cell thickness, 0.9 mm.

fiable matter (0.30 g.) in hexane was passed through a column of a 1:1 mixture of magnesia and celite (75 g., 18 × 3.4 cm.). After development with hexane, five clearly defined zones appeared, the first the most pronounced. When this band separated from the rest of the pigments, the column was removed and the first band (Group I) was cut out from the rest of the bands (Group II). The pigments from the two sections were eluted with 10% methanol in ethyl ether and filtered through a Buchner funnel (No. 42 Whatman paper). The solvent was removed at reduced pressure and replaced with hexane and benzene for Groups I and II, respectively.

After drying the solutions over anhydrous sodium sulfate for 2 hr. (in refrigerator), the carotenoids were rechromatographed on magnesia-celite columns (10 × 1.9 cm.). When Group I was developed with 2% acetone in hexane, only one uniform band (Fraction 1) was formed, indicating that this fraction was chromatographically homogeneous. When Group II was developed with benzene, a small band moved in front and was eluted with this solvent (Fraction 2). When no more pigment was eluted with benzene, ethyl ether was added to wash the benzene from the column. Ethyl ether did not elute any of the bands from the column. When elution was subsequently carried out with 10% methanol in ethyl ether, three distinct bands developed—yellow, orange, and purple, in order of elution. When these three bands were separated, the column was removed and each band was cut out and eluted with 10% methanol in ethyl ether (Fractions 3, 4, and 5). Each one of these fractions, upon rechromatographing on magnesia, gave a single band, indicating its homogeneity.

Characteristic absorption curves (log. absorbance *vs.* wavelength) were obtained, with a Beckman Model DU spectrophotometer, for each of the above fractions dissolved in hexane. The curves (Figure 3) are drawn to coincide at

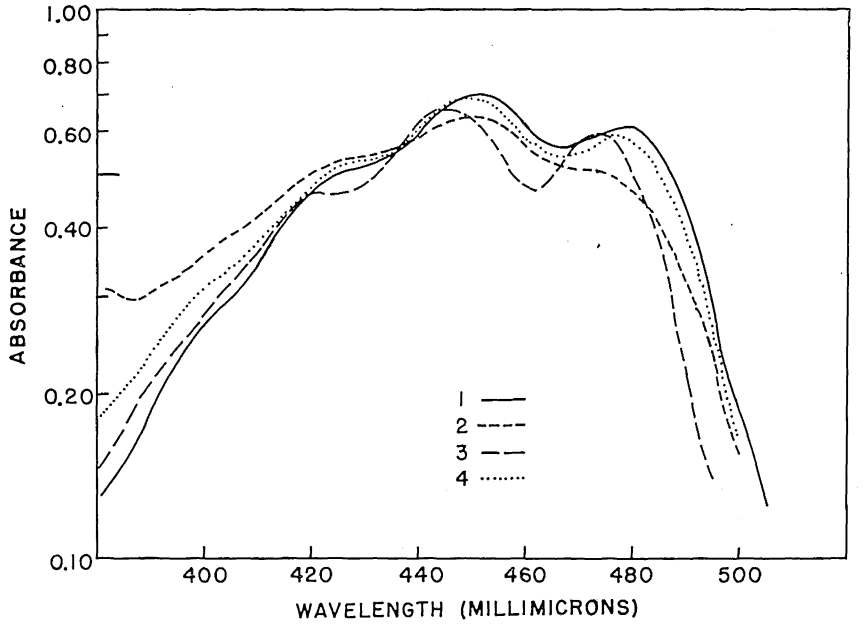


FIG. 3. Absorption spectra of milk fat carotenoid fractions in hexane solution. 1—beta-carotene fraction, 2—neo-beta-carotene fraction, 3—lutein fraction, and 4—zeaxanthin fraction.

437 mμ, which is one of the coincident points for the carotenoids *(24)*. The curves for Fractions 1, 2, and 4 exhibit maxima and minima characteristic *(22)* of the beta-carotene type of molecular structure (λ max. at 450 and 478 mμ, λ min. at 466.5 mμ); whereas, the curve for Fraction 3 has the alpha-carotene structure (λ max. at 422, 446, and 474 mμ; λ min. at 428 and 461.5 mμ). Fraction 1 has the same absorption spectrum as beta-carotene *(24)*. The spectra of Fractions 3 and 4 correspond to those of lutein (xanthophyll, a di-hydroxy alpha-carotene) and zeaxanthin (a di-hydroxy beta-carotene), respectively *(17, 22)*. The hydrocarbon character of Fractions 1 and 2 and the alcohol character of Fractions 3 and 4 were established by the partition test with 85 and 95% methanol *(20)*. Fraction 2 has the same absorption characteristics as neo-beta-carotene *(24)*. This pigment either is present naturally or is produced by alkali and heat isomerization in the saponification step. Fraction 5 exhibited a nondescript absorption spectrum and was probably a carotenoid oxidation product. A quantitative determination of the carotenoids of milk fat showed that beta-carotene (Fraction 1) constituted 85% of the total, which agrees with reported values *(6, 13, 18, 24)*.

The results show that cholesterol can be separated from the unsaponifiable matter of milk fat by adsorption chromatography on alumina or magnesia. Magnesia was effective in separating the carotenoids of milk fat. Further studies are in progress on the separation of milk lipids by chromatographic and countercurrent distribution techniques.

ACKNOWLEDGMENT

Dr. N. K. Freeman, Division of Medical Physics, University of California, Berkeley, California, recorded the cholesterol infrared absorption curves.

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MANUFACTURING COTTAGE CHEESE WITH PAIRS OF SINGLE-STRAIN CULTURES OF LACTIC STREPTOCOCCI

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SUMMARY

Twenty single-strain cultures of lactic streptococci, with different bacteriophage sensitivities, were isolated, studied, and used successfully in manufacturing cottage cheese commercially. Three considerations in arranging the cultures in pairs for use in manufacturing cheese were acid production, antibiotic resistance, and strain compatibility. Four pairs (eight cultures) were used successively in a four-day rotation; each week one pair was replaced by fresh samples of the same cultures. The six reserve pairs (12 cultures) were held in lyophilized form for substitution when examination of composite samples of whey at 2-wk. intervals indicated the presence of a bacteriophage active against one of the cultures in use.

The following advantages resulted from using the pairs of single-strain cultures as described: *(1)* Bacteriophage action was avoided; *(2)* floating curd was eliminated; *(3)* losses in labor and skimmilk from retarded production of acid were decreased; *(4)* cottage cheese quality was more uniform, and *(5)* yield of cottage cheese from skimmilk was increased slightly.

The cultures commonly used in the manufacture of cultured dairy products usually contain several strains of *Streptococcus cremoris* or *Streptococcus lactis* and strains of *Leuconostoc citrovorum* and/or *Leuconostoc dextranicum*. Judged by sensitivity to bacteriophages, some or all of the strains (or types) of lactic streptococci in one particular mixed-strain culture may be the same as those in a mixed-strain culture under a different label *(4)*. One culture, therefore, may breed bacteriophages active against some or all of the strains of lactic streptococci in a different culture to be used subsequently.

This study was undertaken to investigate the usefulness of rotating pairs of single-strain cultures of lactic streptococci in making cottage cheese. Previous results have shown that adding a bacteriophage active against one of two unrelated fast acid–producing strains to a mixture of the two in equal amounts does not appreciably alter total rate of acid production during the first propagation, despite the lysis of one strain *(2)*. A different report *(4)* indicates that the number of strains of lactic streptococci that are different (judged by sensitivity to bacteriophages) and suitable for use in manufacturing cultured dairy products may be small enough to limit seriously the variety of mixed-strain cultures that can be prepared. Further, the value of mixed-strain cultures is limited by the possibility of strain domination *(3, 7, 9, 12)* and the possibility that bacteriophage sensitivities may change after bacterial strains are mixed *(6)*.

Single-strain cultures of *S. cremoris* and *S. lactis* have served satisfactorily in making Cheddar cheese in New Zealand *(15)* and Australia *(8)*. The manu-

Received for publication September 3, 1957.

facture of cottage cheese of good flavor, like the manufacture of cultured butter and buttermilk, has been considered to require the use of species of *Leuconostoc*. Results presented in this paper indicate that these species are not essential in the manufacture of cottage cheese of high quality by current methods.

METHODS AND MATERIALS

Culture sources and isolation. Single-strain cultures of lactic streptococci and bacteriophages active against them were obtained from the Dairy Research Institute of New Zealand and the Commonwealth Scientific and Industrial Research Organization of Australia. The cultures were purified by colony isolation. Additional cultures were isolated from commercial mixed-strain cultures and raw milk. The plating medium was modified trypticase soy agar *(10)*.

Propagating cultures. Cultures were propagated daily (six days per week) in skimmilk fortified with 0.75% nonfat milk solids and autoclaved at 121° C. for 13 min. The inoculum was five drops of culture per 33 ml. of fortified skimmilk. Inoculated bottles of the culture medium were incubated at 22° C. for 16 hr. and then refrigerated. Skimmilk was examined for inhibitors by a test using *Streptococcus thermophilus* and *S. cremoris (5)*. Skimmilk that contained inhibitors was not used for culture medium or activity testing.

After it was found that the sensitivities of single-strain cultures to bacteriophages occasionally changed under daily propagation *(6)*, all of the cultures that were to be used commercially were lyophilized.

Determining bacteriophage sensitivity. The sensitivities of cultures to whey filtrates of the available bacteriophages were determined in litmus milk. Tubes of litmus milk inoculated with bacteriophage filtrates and one drop of culture were incubated at 32° C. for 8 hr. Each was compared to a control tube inoculated only with the same culture. Bacteriophages that did not permit typical reduction and coagulation were considered active.

Determining culture activity. As a preliminary requirement, cultures (following isolation and from two to seven daily propagations in tubes of litmus milk) were expected to coagulate the litmus milk during incubation for 16 hr. at 22° C. Those that passed this preliminary test and had differing bacteriophage sensitivities were propagated for seven days in fortified skimmilk before further testing. Then 5% of fresh culture was inoculated into pasteurized skimmilk and incubated at 31.5° C. in a thermostatically controlled water bath. After 3.75 hr., it was checked frequently, with a pipette, and the time of coagulation recorded. Titratable acidity was determined after 4.5 hr. Final decision as to the value of each promising culture was based on making an experimental vat of cottage cheese.

Arranging the cultures in pairs. Cultures that produced acid satisfactorily and that were different in bacteriophage sensitivity were arranged in pairs for use in cottage cheese plants. The cultures were so arranged as to contain in each pair one very fast acid–producing culture. A mixture of the two paired cultures was checked for ability to coagulate litmus milk in 8 hr. at 32° C. in the presence of each of the two homologous bacteriophages. This procedure eliminated the

possibility that both cultures might later be inhibited by one contaminating bacteriophage *(1, 11, 16)*. Two additional considerations in arranging the cultures in pairs were antibiotic sensitivity and strain compatibility.

Determining antibiotic sensitivity. The sensitivities of the cultures to penicillin and aureomycin in sterile 10% reconstituted skimmilk were determined in sterile screw-cap culture tubes. Graduated amounts of each antibiotic diluted to 10 ml. with skimmilk were inoculated with 1 ml. of diluted culture (1:10 in skimmilk). Inoculated tubes were observed after they had been incubated for 16 hr. at 22° C. The smallest amount of an antibiotic that prevented coagulation was recorded as the sensitivity for that culture. One culture comparatively resistant to antibiotics was included in each pair, when possible.

Determining strain compatibility. Equal amounts (two drops) of the cultures of a pair were inoculated into autoclaved skimmilk and incubated for 16 hr. at 22° C. Activity was determined at 31.5° C. in pasteurized skimmilk inoculated with 5% of the mixture, with and without five drops of each homologous bacteriophage filtrate. Where addition of a bacteriophage caused considerably less acid to be produced, the strain of bacteria sensitive to that bacteriophage was considered to predominate. Only one homologous bacteriophage was available for use in testing some mixtures, because some of the strains of bacteria were resistant to all the available bacteriophages. For those mixtures, a slight retardation resulting from lysis of the sensitive strain was considered to indicate that the resistant strain was not dominant.

Using cultures commercially. Before the pairs of single-strain cultures were tested in a cottage cheese plant, two, and sometimes three, composite whey samples were obtained and checked for bacteriophages active against the cultures. Then four pairs of lyophilized cultures were selected and supplied, with instructions for daily rotation and a request for subsequent whey samples. Samples taken at 2-wk. intervals, beginning one week after the cultures were put into use, usually were considered sufficient.

Each sample was checked for the presence of bacteriophages active against the cultures being used and those that might be used. The method of checking was the same as that described for determining bacteriophage sensitivity, except that unfiltered cottage-cheese whey was substituted for bacteriophage filtrate.

When whey analysis revealed the presence of a bacteriophage active against one of a pair of cultures in use, that pair of cultures was replaced by a different pair, except in instances that will be discussed.

The eight cultures in use were routinely replaced by fresh samples of the same cultures once a month (one pair each week) to guard against changes in bacteriophage sensitivity *(6)*. To avoid strain domination *(3, 7, 9, 12)* the cultures of a pair were maintained separately until the manufacture of cottage cheese. Then equal amounts of the two bulk starters were added to vats of skimmilk.

RESULTS AND DISCUSSION

Culture selection. The 20 cultures (arranged in ten pairs) that were found satisfactory for use in making cottage cheese are listed (Table 1). All of the 20

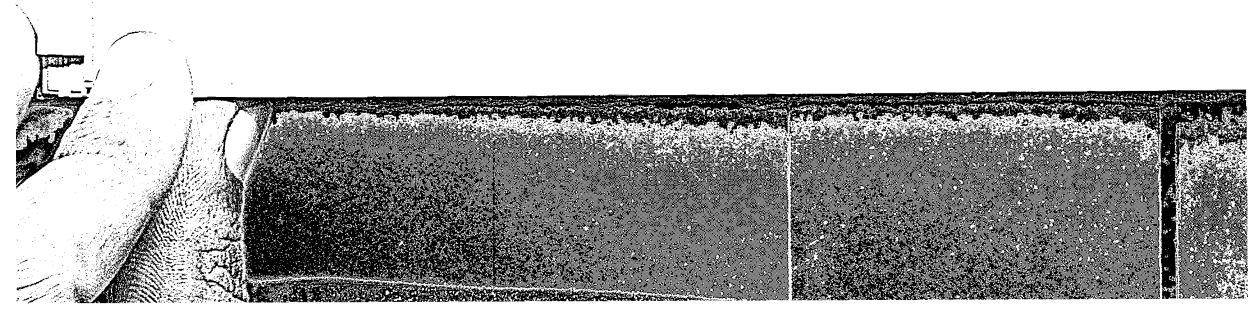


TABLE 1

Pairs of single-strain cultures that served satisfactorily in making cottage cheese

Pair	Culture	Origin		Bacteriophage sensitivity	Antibiotic sensitivity [a]	
		Culture	Source		Penicillin G potassium	Aureomycin HCl
					(units/ml)	*(γ/ml)*
1	A	KH	New Zealand	kh	0.28	0.12
	B	4B	Commercial	Resistant [b]	0.14	0.12
2	C	C3	Australia	c3	0.18	0.18
	D	C13	New Zealand	c13	0.22	0.12
3	E	HP	Australia	hp	0.16	0.18
	F	KH	New Zealand	Resistant [b]	0.26	0.15
4	G	Culture	Commercial	Resistant [b]	0.24	0.12
	H	C6	Australia	c6	0.26	0.18
5	I	C11	Australia	c11	0.24	0.15
	J	Culture	Commercial	c8	0.24	0.12
6	K	ML3	New Zealand	Resistant [b]	0.24	0.18
	L	R1	Australia	r1	0.16	0.15
7	M	Culture	Commercial	Resistant [b]	0.24	0.12
	N	C2	Australia	c2	0.30	0.21
8	O	C7	Australia	c7	0.24	0.21
	P	Culture	Commercial	4b	0.12	0.15
9	R	Culture	Commercial	15c	0.18	0.15
	S	Culture	Commercial	ab3	0.20	0.12
10	T	C2	Australia	Resistant [b]	.....	.....
	W	US3	Australia	us3	0.32	0.18

[a] Two determinations were made. The smallest amount of antibiotic that prevented coagulation is reported.

[b] These cultures were resistant to a mixture of all of the available bacteriophages.

cultures except H, K, N, and T were found to be *S. cremoris,* on the basis of neither growing at 40° C. nor producing ammonia from L-arginine. Cultures H, K, N, and T were *S. lactis.* Six of the 20 were resistant to a mixture of all the available bacteriophages. These six cultures were isolated from different sources and it is supposed that they are different. The possibility that more than one of the six might later be found sensitive to one bacteriophage could not be excluded. The 20 cultures were selected from 84 cultures studied—20 from New Zealand and Australia, 42 isolated from raw milk, and 22 isolated from commercial mixed-strain cultures. Isolations were made from 42 samples of raw milk and 18 commercial cultures. Of the 84 cultures, all but the 20 listed in Table 1 were discarded, because they were related in bacteriophage sensitivity to the cultures retained or because they produced lactic acid too slowly for making cottage cheese satisfactorily. All of the cultures isolated from raw milk were discarded. Those that produced acid satisfactorily were related in bacteriophage sensitivity to the cultures retained.

The activity test eliminated most of the cultures whose acid production was unsatisfactory. Cultures were usually unsuitable for use in making cottage cheese if they required longer than 4.5 hr. for coagulation and had titratable acidities lower than 0.62%. Under daily propagation, cultures usually remained either fast or slow, with only slight day-to-day variations in activity. However, in a few cases, fast cultures gradually became less active (over several days to 3 or 4 wk.) and slow cultures became fast. Improvement in the activity of a slow culture was usually accompanied by a change in bacteriophage sensitivity *(6).*

Slow cultures were not lastingly improved by changing the usual propagation procedure in the following respects: increasing inoculum, decreasing inoculum, transferring every 12 hr., incubating at 32° C. for 8 hr., or using nonfat milk solids (11%) as culture medium.

Strain compatibility. Some mixtures of two cultures produced considerably less lactic acid when one homologous bacteriophage was added. This finding substantiated reports that some single-strain cultures will dominate others in mixtures within one or two propagations *(7, 9)*. Culture C dominated each of the 19 cultures, except D. Though C was normally compatible with D, lysis of C delayed coagulation about 2 hr. on both of two occasions when the two cultures were started from the lyophilized state, propagated only twice separately, mixed, propagated once as a mixture, and tested for domination. Because of the dominance findings, recouplings and tests were conducted to rearrange the 20 cultures in pairs that would be compatible for one propagation. The resultant pairings are shown (Table 1). When these pairs were grown as mixtures for one propagation preliminary to use in tests, domination did not occur (shown in Table 2).

Cheese plants that used the cultures experimentally maintained the cultures of each pair separately until the manufacture of cottage cheese. This procedure was recommended in preference to mixing the cultures of a pair in making bulk starter. But the results in Table 2 indicate that the latter method should give satisfactory results in plants that do not have and can not obtain two bulk starter vats.

Cottage cheese yield. Vats of cottage chese curd made with pairs of single-strain cultures of lactic streptococci were observed to have clearer whey than vats made with commercial mixed-strain cultures. At the same pH, the per cent titratable acidity of the clear whey was about 0.03 lower than that of the more turbid whey. To determine whether the single-strain cultures gave greater recovery of solids, comparison vats using the same skimmilk with both culture types were made each day for four consecutive days. Five commercial mixed-strain cultures were used, a different pair each day. (Two mixed-strain cultures were used each day because this was the usual plant procedure.) The vat receiving single-strain cultures also received a different pair each day.

In the experiment, the single-strain cultures yielded an average of 1.3% more cottage cheese (not attributable to differences in moisture content) than the mixed-strain cultures (Table 3). There were daily variations in the amount of increase, indicating that its magnitude can be determined only by comparing the culture types on a much larger scale.

The results do not indicate the reason for the clearer whey and slightly increased recovery of solids. Possibly, it is the result of more rapid and/or more uniform coagulation, or cheese curd may shatter less when it does not contain the small amounts of carbon dioxide normally produced by mixed-strain cultures containing species of *Leuconostoc*.

Cottage cheese flavor. Flavor comparisons were included in the above test. Also, in a different plant, one vat was made with single-strain cultures and the remaining vats with commercial mixed-strain cultures daily for 1 mo. In both

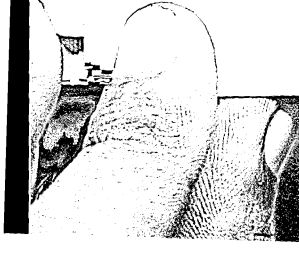


TABLE 2

Influence of bacteriophages on the activities [a] of pairs of single-strain cultures that had been grown as mixtures for 16 hr. at 22° C.

Pair	Bacteriophage added	Titratable acidity [b]	
		4 hr.	5 hr.
		(%)	
1	None	0.63	0.75
	kh	0.57	0.71
2	None	0.59	0.72
	c3	0.50	0.67
	c13	0.46	0.64
3	None	0.62	0.70
	hp	0.53	0.69
4	None	0.59	0.71
	e6	0.53	0.68
5	None	0.67	0.72
	c11	0.54	0.72
	e8	0.53	0.64
6	None	0.62	0.70
	r1	0.50	0.66
7	None	0.64	0.74
	e2	0.59	0.74
8	None	0.68	0.69
	c7	0.61	0.72
	4b	0.60	0.71
9	None	0.60	0.74
	15c	0.49	0.66
	ab3	0.49	0.68
10	None	0.66	0.72
	us3	0.49	0.66

[a] Activity was determined at 31.5° C. in pasteurized skimmilk inoculated with 5% of a mixture, with and without addition of each homologous bacteriophage.

[b] The average values for two experiments are reported.

cases, flavor comparisons were made by representatives of the companies and members of the University's Department of Dairy Industry staff, separately. Included in each group were two or more experienced judges of cottage cheese.

Consideration had been given to the possibility that using pairs of single-strain cultures might make it necessary to add small amounts of starter distillate or biacetyl. But the flavor comparisons indicated that such additions are not required. On the contrary, the results revealed that cottage cheese of high quality and taste appeal can be made with single-strain cultures of lactic streptococci with no *Leuconostoc* involved. In most cases the judges could not distinguish between the lots of cheese made with pairs of single-strain cultures and those made with commercial mixed-strain cultures. Occasionally, slight differences were detected, sometimes favoring single-strain cultures and sometimes favoring mixed-strain cultures. These differences were attributed to normal variations in manufacturing procedure, rather than to culture type.

TABLE 3

Increased yields of cottage cheese with pairs of single-strain cultures

Trial [a]	Culture type	Skimmilk	Cottage cheese [b]	Increase
		(lb.)		
1	Pair	6,880	1,811	
	Commercial	6,880	1,772	39
2	Pair	6,880	1,791	
	Commercial	6,880	1,782	9
3	Pair	6,880	1,601	
	Commercial	6,880	1,575	26
4	Pair	7,310	1,950	
	Commercial	7,310	1,931	.19

[a] The two vats used in a trial received quantities of the same skimmilk and the cottage cheese was made by regular plant personnel.

[b] Per cent solids of the cottage cheese was determined by the Mojonnier method. For purposes of comparison, the actual weight was corrected so as to represent cottage cheese containing 20% solids.

In each of the above comparisons, the manufacturers, following their usual procedure, added small amounts of commercial mixed-strain culture to the creaming mixtures—to increase the shelf-life of cottage cheese and, at above-normal storage temperatures, to produce sourness rather than permit surface spoilage. The following observations indicate that this practice did not influence the judgments of cottage cheese quality: *(1)* The cottage cheese had good flavor soon after the creaming mixture was added. *(2)* Flavor was the same when the amount of mixed-strain culture was decreased from about 700 ml. to 1 ml. per 500 gal. of creaming mixture. *(3)* Flavor was the same when a single-strain culture was added to the creaming mixture in place of the commercial mixed-strain culture. *(4)* Three of the plants that adopted the pairs of single-strain cultures make high-quality cottage cheese without adding any culture to creaming mixtures.

After the flavor comparisons had been made, several of the single-strain cultures were analyzed, by steam distillation, for the presence of biacetyl. The amounts were negligible, less than 0.01 p.p.m.

The compounds produced by species of *Leuconostoc,* particularly biacetyl, possibly have become unnecessary, because of a change in the desired characteristics of cottage cheese. Economic factors and consumer acceptance have established general use of the short-set method of manufacturing cottage cheese curd. Consumer demand has recently increased considerably the ratio of small to large curd. By current methods, considerable lactic acid and much of the biacetyl are washed from cottage cheese curd. The resulting bland, tender curd is flavored by the addition of cream and salt to give a clean-tasting, creamy, mild cottage cheese. When made from high-quality ingredients, this cottage cheese apparently has greater consumer appeal, though it usually does not have the detectible biacetyl odor of the more acid, highly flavored cheese manufactured several years ago.

Number of bacteriophage particles. On two occasions when bacteriophages were found in samples of whey from one cottage cheese plant, the sensitive cultures were not immediately replaced. Instead, to determine the influence of culture rotation on the number of bacteriophage particles in the cheese whey, samples of whey were examined daily. The sanitary precautions used to avoid bacteriophage contamination in this plant were above average, including the use of a chlorine solution (at least 400 p.p.m.) on vats and equipment, immediately before filling the vats with skimmilk. But two vats of curd were made each day with hold-over bulk starter. The plant was requested to exclude those two vats in gathering whey samples, but the same cheese-making equipment was used in all vats. This practice undoubtedly influenced the numbers of bacteriophage particles in the wheys.

Each use of a sensitive culture resulted in a large number of bacteriophage particles in the corresponding whey (Table 4), but there was no evidence of slow acid production in manufacturing cheese, since each affected pair contained one resistant culture. Possibly, there were occasions when the resistant cultures produced almost all the lactic acid.

For one or two days prior to each rotation of a pair that included a sensitive culture, active bacteriophage particles were not detected. This result indicates that the rotation of cultures was helpful in minimizing the number of bacteriophage particles present in the cheese plant. It also shows that manufacturers using the cultures should include in the samples of whey, taken at 2-wk. intervals for examination, some whey from each of four consecutive days (some whey from each pair of cultures being used).

On neither occasion was the bacteriophage eliminated from the cheese plant, emphasizing the extreme difficulty—if not impossibility—of completely

TABLE 4

Influence of culture rotation on the titers of bacteriophages in cottage cheese whey

Trial 1			Trial 2		
Day	Pair of cultures used in making cottage cheese	No.[a] bacteriophage particles/ml active against Culture D[b]	Day	Pair of cultures used in making cottage cheese	No.[a] bacteriophage particles/ml active against Culture A[b]
Monday	CD	100,000,000	Tuesday	AB	10,000,000
Tuesday	IJ	100,000	Wednesday	IJ	1,000
Wednesday	GH	1	Thursday	GH	1
Thursday	EF	None detected	Friday	EF	None detected
Friday	CD	100,000,000	Saturday	AB	10,000,000
Saturday	IJ	10	Sunday	IJ	1,000
Sunday	GH	None detected	Monday	GH	None detected
Monday	EF	None detected	Tuesday	EF	None detected
Tuesday	CD	100,000,000	Wednesday	AB	10,000,000
Wednesday	IJ	10,,000	Thursday	IJ	1,000
Thursday	GH	1	Friday	GH	1
Friday	EF	None detected	Saturday	EF	None detected

[a] Approximate number determined by the limiting active dilution method.

[b] The wheys did not contain bacteriophages active against the seven other single-strain cultures.

eliminating a bacteriophage from a plant if a sensitive culture is used, even occasionally. The results justify replacement of a culture pair whenever a bacteriophage active against one of the cultures is found in whey from a plant. Since ten pairs of single-strain cultures are available for use and only four pairs are used in the rotation plan, a plant can count on considerable time for sanitary measures to eliminate a bacteriophage before a culture that has been replaced will be used again.

Rotating pairs of single-strain cultures of known different bacteriophage sensitivities is only supplementary; it cannot replace sanitary measures to safeguard against contamination with bacteriophages and to destroy any that are present. It supplements sanitary measures by allowing a bacteriophage that invades the cheese vats to multiply every fourth day only (four pairs of cultures being used and seven of the cultures being resistant). Vats, floors, walls, etc. will be sanitized four times before multiplication of the bacteriophage in the cheese vats is again possible.

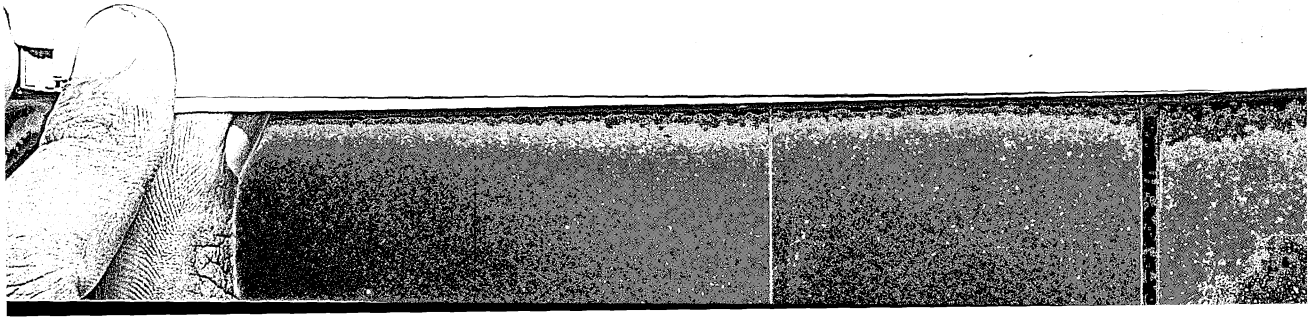
Commercial application. At first, single-strain cultures were used experimentally in but one plant. At the request of operators interested in the success of the method, the experiment was extended to six more plants. Three of the plants have used the cultures for over 1 yr. Total cottage cheese production in the seven plants is about 1.5 million pounds per month. With the new procedure, the plants have reported greater uniformity in curd and production time. Before the change, outbreaks of floating curd from gas produced by commercial mixed-strain cultures *(13)* occurred frequently in some of the plants. These ceased immediately after the change. Three of the plants had been having difficulty with slow production of acid caused by the action of bacteriophages. One plant lost seven vats of skimmilk the month before starting use of the single-strain cultures; each of the other two lost three vats in 1 wk. None of the plants has had difficulty with slow production of acid caused by bacteriophages since adopting the single-strain cultures, though bacteriophages occasionally have been detected in five of the plants.

Slow production of acid caused by antibiotics and certain other inhibitory substances in milk is not prevented by using cultures of known bacteriophage sensitivity. In certain seasons and in some areas, tests may still be required to avoid using milk that contains such inhibitors.

The primary aim of this study was to develop a procedure that will simplify the problem of ascertaining whether slow production of acid is caused by bacteriophages—a procedure that will permit predicting and preventing slow production of acid caused by bacteriophage action. Smith *et al. (14)*, after tracing to bacteriophages 21 of 23 cases of slow production of acid, concluded, "The evidence suggests that in many instances it might be appropriate to eliminate bacteriophage as the cause of pronounced lactic streptococcus starter failure before attributing responsibility to other agents."

ACKNOWLEDGMENTS

The author wishes to express appreciation for the cultures and bacteriophages contributed by Dr. H. R. Whitehead, of the Dairy Research Institute, Palmerston North, New Zealand,



and by Mr. J. Czulak, of the Commonwealth Scientific and Industrial Research Organization, Highett, Victoria, Australia, and for technical assistance from Mr. G. D. Young, of the Dairy Products Laboratory, San Francisco, California, and Mr. G. S. Mason, of the Borden Company, Gustine, California.

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VARIABILITY IN RESPONSE OF LACTIC STREPTOCOCCI TO STIMULANTS IN EXTRACTS OF PANCREAS, LIVER, AND YEAST[1]

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SUMMARY

This study was directed to ascertaining the cause of different growth responses by starter streptococci in milk and in milk supplemented with certain stimulatory extracts. Aqueous extracts of pancreas tissue, when added to milk, were stimulatory for the development of certain species of the family *Lactobacteriaceae,* but not for representative species of milk-spoilage types of bacteria. Litmus milk agar bioautographs of paper chromatograms showed the presence of multiple stimulatory components in the pancreas extract. Preliminary tests indicated that these components were peptides. Extracts of liver and yeast also contained these same stimulatory factors. Various strains of *Streptococcus lactis* and mixed strain streptococci starter cultures differed in their response to the individual stimulatory components. All cultures responded to certain components, whereas the response to others was irregular. There was evidence of an inverse relationship between the number of components to which a culture responded and the rate at which the culture grew in milk.

The growth of lactic acid streptococci in milk has been shown to be stimulated by the addition of various crude extracts of plant and animal tissues, and by enzymatic hydrolysates of proteins *(1, 2, 3, 5, 7)*. Slow cultures have been reported to be stimulated more than fast cultures by the presence of certain additives in milk; also, different crude extracts varied in the extent to which they stimulated lactic streptococci in milk *(1, 2)*.

A large amount of the stimulation effected by tryptic digests of proteins has been found to reside in the impurities in the enzyme preparation when crude forms of the enzyme were used to prepare protein digests *(7)*. Pancreas glands were presumed to be the source of these stimulants in crude trypsin preparations, because the stimulants from both sources appeared to be identical by chromatographic analyses *(3)*. Two stimulants in extracts of pancreas glands, which were active in presumably complete semisynthetic media and in milk, were found to be peptides; their amino acid content has been determined *(8, 10)*.

The response of various cultures of lactic streptococci, when growing in milk, to the individual stimulatory components in crude extracts of animal and plant tissue, has not been reported. Information of such nature could be important in the proper selection and use of cultures from milk fermentations, and possibly lead to a clarification of the role of bacterial stimulants in milk.

The present study was made to observe any variability in the response of single-strain lactic streptococci and mixed-strain starter cultures to different stimulatory components in extracts of pancreas, liver, and yeast.

Received for publication June 20, 1957.

[1] Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station, Raleigh, as Paper No. 821 of the Journal Series.

[2] Dairy Industries Supply Association Fellow.

EXPERIMENTAL PROCEDURE

The cultures used were carried by weekly transfer in sterile litmus milk [nonfat dry milk solids (NFDMS) reconstituted to 10% solids]. Fresh 24-hr. cultures were used as inocula throughout these studies. *Pseudomonas fragi* and *Leuconostoc citrovorum* were carried at 20° C. The strains of *Streptococcus lactis* were carried at 32° C. *Escherichia coli* and *Bacillus subtilis* were incubated at 35° C. The single-strain lactic streptococci (No. 2-12 in Table 1) and mixed-strain starters were carried in pasteurized (210° F.—30 min.) 10% NFDMS at 21° C.

The aqueous extract of pancreas tissue was prepared as described by McAnelly and Speck *(8)* and contained 40 mg. solids per ml.

For the growth studies in milk, six tubes containing pancreas extract and six control tubes were prepared for each culture to provide individual tubes of both for each incubation period. Ten milliliters of 15% reconstituted skimmilk was added to each tube and the tubes were sterilized at 121° C. for 15 min. One-half milliliter of sterile (112° C. for 6 min.) pancreas extract and 4.5 ml. of sterile distilled water were added to one set of tubes. Five milliliters of sterile distilled water was added to each of the control tubes. One milliliter of the 24-hr. culture that had been diluted 1.6 : 10 with sterile skimmilk was added to each tube. The tubes were incubated at the temperatures noted above for the different organisms. Plate counts or titratable acidity determinations (measured as lactic acid with 0.1 *N* NaOH) were made after 0, 2, 5, 7, 12, and 24 hr. of incubation. *L. citrovorum* was plated with trypticase soy agar (made from individual ingredients) and *E. coli, P. fragi,* and *B. subtilis* were plated using plate count agar (Difco). All plates were incubated for 48 hr., except in the case of *L. citrovorum,* which required 72 hr. for the development of colonies of sufficient size to count.

The response of different cultures to the various stimulatory components in pancreas extract was studied by paper chromatographic and bioautographic techniques. Paper strips (3.8 by 57 cm.) were spotted with 4.0 mg. of pancreas extract solids across the base line (10 cm. from one end). The strips were developed in butanol, acetic acid, and water (4 : 1 : 5) at room temperature (approximately 25° C.), with descending solvent flow until the solvent was 3-7 cm. from the end of the strip. The developed strips were dried by exposure to a forced draft for 24 hr. at room temperature to remove solvents.

Stimulatory areas on the chromatographs were detected by a modification of the bioautographic technique of Kennedy *et al. (6)*. Nonfat dry milk solids were reconstituted to 18% solids with distilled water in 125-ml. quantities and autoclaved at 121° C. for 15 min. The sterile milk was refrigerated for 12 hr. to allow the oxidation-reduction potential to return to normal. It was tempered to 45-50° C., 5 ml. of a 20% sterile litmus solution and 125 ml. of sterile 4% washed agar were added, and the medium was seeded with 2.5 ml. of a 24-hr. litmus milk culture of the test culture. The inoculated medium was poured into a sterile 23 by 38 by 5 cm. pyrex dish, that was fitted with a glass cover, and was allowed to solidify. One-half (lengthwise) of a chromatogram was placed on the

surface of the hardened medium for 10 min., then removed. The bioautograph plate was then incubated at 32° C. The plates were observed for the appearance of active areas during a period of 3.5-7 hr. after the beginning of the incubation period. The active areas, which resulted from accelerated growth, appeared as white spots of reduced litmus against a blue-pink background.

RESULTS

Stimulatory activity of pancreas extract in milk. The effect of pancreas extract was tested on cultures typical of those in starter cultures, as well as on some undesirable organisms commonly found in dairy products. Pancreas extract was found to stimulate acid production of both strains of *S. lactis* (Figure 1).

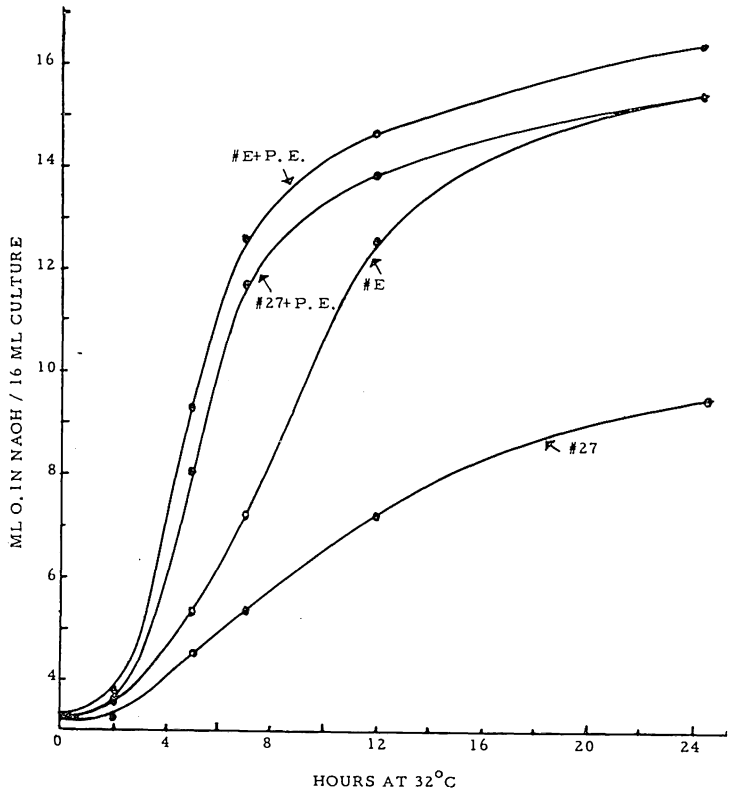


FIG. 1. Acid production by a slow (27) and fast (E) culture of *S. lactis* in skimmilk containing 1.25 mg/ml of pancreas extract solids (P.E.).

S. lactis E was not stimulated so greatly as was *S. lactis* 27, the latter being a characteristically slow culture (i.e., produces acid slowly in milk). These results support the data of Anderson and Elliker *(1)* and Garvie and Mabbitt *(2)*, who found that slow starter cultures responded more to stimulants than did fast cultures.

Growth of the culture of *L. citrovorum,* as measured by colony counts, also

TABLE 1

Response of single and mixed cultures to components in different crude extracts

Culture	Rf values of stimulatory zones on chromatograms of													
	Pancreas extract				Liver extract					Yeast extract				
Streptococcus lactis E		0.27	0.43			0.28	0.34	0.44			0.27	0.34	0.42	
Streptococcus lactis 27	0.05	.28	.42	0.58	0.05	.28	.35	.43	0.58	0.02	.26	.36	.41	0.58
Single Strain 2	.04	.27	.42		.03	.23	.31	.38		.01	.25	.32	.40	
Single Strain 3	.02	.26	.42		.02	.27	.34	.42		.02	.25	.31	.40	
Single Strain 4	.02	.24	.39		.08	.27		.40		.02	.26	.35	.40	
Single Strain 5	.02	.27	.42		.03	.27	.34	.43		.02	.27		.43	
Single Strain 6	.03	.27	.41		.03	.25	.34	.43		.02	.26	.34	.42	
Single Strain 7	.03	.26	.40		.03	.23	.33	.41		.02	.23	.31	.40	
Single Strain 8	.02	.25	.41		.02	.24	.32	.38		.02	.24	.34	.40	
Single Strain 9	.02	.26	.41		.02	.25	.33	.41		.02		.35	.42	
Single Strain 10	.03	.26	.41		.02	.27		.43		.02	.25		.41	
Single Strain 11	.04	.25	.41		.03	.26		.42		.02	.27		.42	
Single Strain 12	.02	.27	.41		.03	.27	.33	.40		.02	.24	.30	.38	
Mixed Strain B_3	.02	.26	.42			.25	.33	.42			.26	.34	.41	
Mixed Strain $F!_3$	.02	.27	.40		.04	.28	.36	.44		.02	.28	.33	.40	
Mixed Strain C_2	.02	.25	.41		.02	.24	.31	.41		.02	.24	.34	.40	
Mixed Strain 1823		.25				.27		.40			.28		.44	
Average	0.03	0.26	0.41	0.58	0.03	0.26	0.33	0.41	0.58	0.02	0.26	0.33	0.41	0.58

was stimulated by the addition of the pancreas extract. Conversely, *E. coli*, *P. fragi*, and *B. subtilis* were not stimulated by the addition of pancreas extract to milk; odor and appearance of the cultures also indicated no difference in the pancreas-supplemented cultures.

These results indicate that the pancreas extract was stimulatory for cultures normally present in lactic streptococci starters, but not for undesirable types of bacteria encountered in milk products.

Variability in stimulatory properties of pancreas extract for S. lactis. A reasonable explanation for the difference in response by the two cultures appeared to be that pancreas extract may have contained more biologically active components for the slow culture (*S. lactis* 27) than for the faster one (*S. lactis* E). In order to study this more specifically, the paper chromatographic and bioautographic techniques were employed. *S. lactis* 27, the slower-growing culture, responded to four areas on the chromatograph (Table 1). These areas were at Rf 0.05,[3] 0.28,[3] 0.42, and 0.58. In contrast, *S. lactis* E responded only to components at Rf 0.27 and 0.43. A color reaction with ninhydrin also was obtained at each of the four Rf values. The factors at Rf 0.05 and 0.28 have been found to be peptides *(8, 10)*, and preliminary tests indicated that the factors at the two other areas also are peptides.

Stimulatory factors in various extracts for S. lactis *and mixed strain starters.* After observing the differences in response by the fast and slow cultures to factors in pancreas extract, the study was extended to other cultures and to other extracts. Pure cultures of lactic streptococci used in preparing commercial starters and several mixed-strain cultures were obtained for use in this part of the study. Chromatograms were prepared of liver extract *(9)* and yeast (Difco) in the same manner as for pancreas extract. Bioautographs of these chromatograms were then prepared using the different single- and mixed-strain cultures of lactic streptococci as inocula. The responses of these cultures to factors in the different extracts are presented (Table 1). Most of the cultures responded to three factors in the pancreas extract. Exceptions were: 1823, a very rapidly growing culture, which responded to one factor; E, a comparatively rapid culture which characteristically responded to two factors, and 27, a slow culture which normally responded to four factors. In the yeast and liver extracts there were four factors to which many cultures responded. Culture 1823, however, again responded to fewer factors than any other culture; its response to the two factors in yeast and liver extract at times was questionable.

It was interesting to note that factors at average Rf values of 0.03, 0.26, and 0.41 were present in all three crude extracts. In the yeast and liver extracts an additional factor at an average Rf value of 0.33 was present and active for most of the cultures. The results (Table 1) indicated that there was a qualitative difference in the response to factors present in the three extracts; thus, certain of these factors regularly elicited a definite response by all the cultures, whereas others were less regularly utilized.

[3] In previous studies *(8, 10)*, chromatographs were developed at higher temperatures and the factors at Rf 0.03 and Rf 0.26 were located at Rf 0.05 and Rf 0.32, respectively.

DISCUSSION

The present study has confirmed earlier studies in which the acid production by lactic streptococci has been reported to be stimulated by the addition of certain crude extracts to milk. The data showed that each of the three extracts used contained multiple stimulants for most of the cultures of lactic streptococci. Four factors (at Rf 0.03, 0.26, 0.41, and 0.58) were found in all three extracts; another factor (at Rf 0.33) was present in liver and yeast extracts but not in pancreas extract. Two of the factors (at Rf 0.02 and 0.26) in pancreas extract have been found to be peptides *(8, 9);* presumably, the other factors also are peptides, because they react with ninhydrin and activity is lost on hydrolysis with acid.

There were indications of an inverse relationship between the growth rate of a culture and the number of peptides in the extracts to which the culture showed response. Possibly, this was a reflection of the proteolytic activities of the culture. Faster cultures presumably obtain needed peptides by hydrolysis of milk proteins and thus show a response to fewer preformed peptides furnished from outside sources. This is consistent with the report of Harriman and Hammer *(4),* who found that there was a direct correlation between the proteolytic activity of cultures of *S. lactis* and the rate with which they grew in milk.

The present study also indicated that the response of cultures to peptides in crude extracts was qualitative. Thus, the factor at the average Rf value of 0.26 was active for all cultures and the one at Rf 0.41 was active for all cultures except 1823, when testing pancreas extract chromatograms. The components at Rf 0.03 and 0.33 were irregularly active, although many cultures responded to both factors. Only *S. lactis* 27, the very slow culture, responded to a factor at Rf 0.58.

It would appear, therefore, that the factors at Rf 0.26 and 0.41 are readily utilizable by most cultures and needed for their optimum growth; the other factors are needed by cultures which are less capable of rapid growth (less proteolytic?) without the addition of these factors in a preformed state. Evidence available indicates that various peptides can be stimulatory to the growth of lactic streptococci, and that the amino acid content of these peptides is similar with respect to certain amino acids *(8).* There is reason to suspect, however, that the configuration of certain amino acids within the peptide molecule may be equally as important in determining the biological activity of the peptide. Further studies are planned, to determine the structure of stimulatory peptides.

There are certain practical implications of this study with respect to the choice of cultures for commercial uses. It would appear that cultures with less dependence on the presence in milk of preformed peptides would be capable of better growth and should be chosen for the making of fermented milk products. However, there may be certain qualities of a culture which would make its use as a starter desirable, in spite of its greater dependence on preformed peptides for most rapid growth. In such an instance, it would be advantageous to supplement milk with a source of the stimulatory peptides, in order to obtain the quali-

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ties desired of the culture and to promote its rapid growth. More information is needed before these assumptions can become tenable.

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A RAPID METHOD FOR DETERMINING THE FAT EMULSION STABILITY OF HOMOGENIZED FLUID MILK PRODUCTS[1]

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SUMMARY

The Babcock centrifuge and fat testing apparatus were used. The method follows: Add 1 ml. of Sudan III to a milk test bottle, then add 17.5 ml. of milk at 110° F. and shake for 15 sec. Add two 10-ml. portions of distilled water at 110° F., shaking for 30 sec. after each addition. Then fill the bottle to between the 7 and 8% marks with distilled water at 110° F., being careful not to shake. Balance bottle in a centrifuge heated to 110° F., using the outside trunnion cups, and spin for 10 min. Read directly from the centrifuge, the cream volume to the nearest 0.1%. Each 0.1% cream volume is about equal to 1.0% difference in the USPHS Index. Sudan III made the cream volume more distinct. Ethyl alcohol carried the dye. With this method small differences can be detected in fat emulsion stability as affected by some processing procedures.

Homogenization is an important process in the dairy industry. Most of the market milk, and all evaporated and sterile milk, is homogenized. The USPHS Index *(6)* is the recognized method for determining homogenization efficiency, but 48 hr. must elapse after processing the milk before the results can be made available. A more rapid method is desirable because a cream plug or cream layer detracts from the palatability and appearance of the product. In many instances, too much reliance has been placed on the pressure of the homogenizer to control cream-rising. The condition of the equipment and the processing procedures also are important *(5)*.

Several methods have been suggested for determining homogenization efficiency. Farrall, Walts, and Hanson *(1)* developed a microscopic method. Snyder and Sommer *(4)* used centrifugal separation, and Maxcy and Sommer *(3)* determined homogenization efficiency of evaporated milk with a centrifugally accelerated fat separation test. They *(3)* stated that the difficulty with the Snyder and Sommer test *(4)* was that the cream layer in a Babcock milk test bottle frequently lacked distinctness, especially when the fat was lightly pigmented.

The purpose of this study was to develop a rapid method to determine homogenization efficiency of market milk, evaporated, and sterile milk, and which could be adapted to routine use.

EXPERIMENTAL PROCEDURE

The Sudan III solution was prepared by adding 3.0 g. of Sudan III (86% dye content) to 100 ml. of 95% ethyl alcohol which was allowed to stand for 24 hr., then filtered.

Received for publication July 30, 1957.

[1] This study was supported by U. S. Public Health Grant No. 1947.

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A Babcock centrifuge with trunnion cups 2 in. deep and regular milk-testing equipment were used.

The test was conducted as follows: Add 1 ml. of Sudan III solution to a Babcock milk test bottle, then add 17.5 ml. of well-mixed homogenized milk previously forewarmed to 110° F. and shake the mixture for 15 sec. Add two 10-ml. portions of distilled water at 110° F., shaking for 30 sec. after each addition. Then fill the test bottle with distilled water at 110° F. to between the 7 and 8% marks, being careful not to shake after this addition of water. It is important to balance the bottles in the centrifuge, using the outside trunnion cups only. Place bottles in a centrifuge, preheated to 110° F. and of known r.p.m., for 10 min. Read the cream column to the nearest 0.1% directly from the machine. Each 0.1% cream volume in this test is about equivalent to 1.0% difference in the USPHS Method. For evaporated milk the same procedure was followed, except that a 9-g. sample was used.

It was necessary to adjust the r.p.m. of different centrifuges to attain a force equivalent to that in an 18-in. wheel revolving at 1,115 r.p.m. (Table 1

TABLE 1

Equivalent speeds required with wheels of different diameters[a]

Diameter of wheel	R.p.m. required
(in.)	
16	1,204
17	1,157
18	1,115
20	1,043
22	983
24	933

[a] Data of Snyder and Sommer *(4)*.

contains r.p.m. data required for wheels of different diameters.) An equivalent level of separation may be obtained with wheels operating at other speeds, provided that the time is varied according to the equation:

$$\frac{S^2}{S_1^2} = \frac{t_1}{t} \tag{1}$$

S = standard r.p.m. for centrifuge

S_1 = actual speed at which centrifuge is running

t_1 = time required at S_1 to equal the effect of S at time of t.

Since time in this method is 10 min., the formula becomes:

$$t_1 = \frac{S_2 \times 10}{S_1^2} \tag{2}$$

RESULTS AND DISCUSSION

The use of Sudan III improved the clarity of the cream column in the neck of the test bottle. Ethyl alcohol was used as a carrier for the dye, but it also hastened the separation of fat globules.

Effect of the angle of centrifuge cups. The outside trunnion cups of the wheel were used to obtain the proper angle. Cream separates faster in a bottle moving at an angle than it does in one moving horizontally, because the globules travel shorter distances before they collide, agglomerate, and hit the wall of the test bottle *(2)*. Figure 1 shows the angle in an 18-in. wheel with cups 2 in. deep,

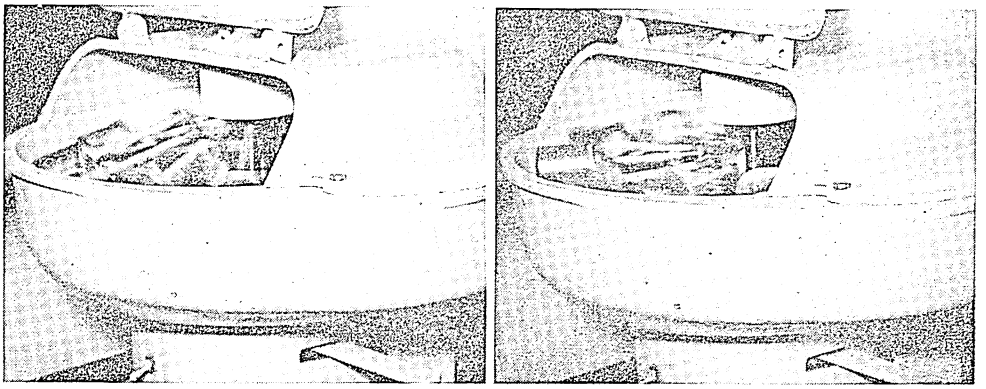


FIG. 1. *Left*: Angle of the test bottles when the outside trunnion cups were loaded. *Right*: Loss of angle when all cups were loaded.

moving at 790 r.p.m., when the bottles were placed in the outside cups only, as compared to loading all cups. By loading only the outside cups, an angle of about 25 degrees was obtained, which was sufficient to obtain readable cream volumes. When all trunnion cups were loaded, no distinct cream volume was obtained within a reasonable time.

The depth of the centrifuge cups also was found to be a factor. Cups 5 in. deep were not satisfactory, because the angle obtained during centrifuging was only from 5 to 6 degrees, which was not sufficient to separate the cream quickly.

Effect of various speeds of centrifuges. Adjusting time, to compensate for the speed of different wheels in accordance with Equation One, resulted in small but definite differences in the cream volumes obtained (Table 2). How-

TABLE 2

Effect of varying the r.p.m. of the wheel (18-in. diameter) on the Centrifugal method

Centrifuge No.	I	II	III	IV	
R.p.m.	700	794	864	1,080	
Time *(min.)*	25.5	20	16.5	10.5	
Homogenization pressure	Cream volume				USPHS Index
(p.s.i.)	(%)				
0	7.6	7.6	7.5	7.6	75.0
500	5.1	5.5	5.2	5.55	44.5
1,000	2.95	4.0	3.3	3.4	28.0
1,500	2.4	2.7	2.45	2.45	24.0
2,000	1.7	2.35	2.05	1.8	20.0
2,500	1.3	1.75	1.85	1.5	14.0

ever, these small differences would not detract from the usefulness of the method for comparative purposes, because they were no greater than the possible error between operators who might determine the USPHS Index.

Comparison of the Centrifugal method with the USPHS Index. Microscopic examination of the fat globules in the neck of the bottle showed that they were from 1 to 6 μ in diameter, averaging about 3.0 to 3.5 μ. It is evident that results obtained with the Centrifugal method agreed closely with the USPHS Index (Table 3).

TABLE 3

Comparison of the Centrifugal method with the USPHS Index on commercial homogenized milks

Source	Centrifugal method [a]	USPHS Index
	(%)	
Dairy A	1.5	14.0
B	1.1	10.1
C	1.0	9.5
D	0.9	8.0
E	0.7	6.5

[a] Each 0.1% cream volume is about equal to 1% difference in the USPHS method.

Estimations on evaporated milk. Samples of evaporated milk collected from different markets in the United States and Canada were tested by the Centrifugal method. From the results (Table 4) it will be noted that the majority of samples had cream volume of not more than 0.1%. In addition, these data show that the most stable or efficiently homogenized milks had a cream volume of from 0.00 to 0.05%.

TABLE 4

Fat emulsion stability in evaporated milk determined by the Centrifugal method

Source	No. samples	Centrifugal method	
		Minimum	Maximum
		(%)	
Company F	8	0.05	0.05
G	6	0.10	0.25
H	4	0.00	0.05
I	4	0.15	0.15
J	2	0.00	0.05
K	2	0.10	0.15
L	2	0.70	0.80

Estimations on sterile milk. Sterile milk is produced in a limited quantity in the United States. One problem with this product is the difficulty of maintaining a uniform dispersion of fat during long storage. The use of ultra-high temperatures of heating partially destroys creaming ability; however, cream-rising occurs in a few days in milks which are inefficiently homogenized. During this study, several batches of sterile milk, heated from 285 to 305° F. and held about 2 sec., were examined. Some milks were homogenized after preheating to 140 or 180° F. and before sterilization; whereas, others were homogenized after sterilization with a special assembly. The data (Table 5) show that the Centrifu-

TABLE 5

Effect of various treatments on fat emulsion stability in sterile milk

Treatment	Centrifugal method	Conversion to USPHS
	(%)	
Preheated to 140° F., homogenized, sterilized	0.25	2.50
Preheated to 180° F., homogenized, sterilized	0.05	0.50
Preheated to 140° F., sterilized, cooled to 160° F., homogenized	0.45	4.75
Preheated to 180° F., sterilized, cooled to 160° F., homogenized	0.50	5.00

gal method can estimate small differences in the degree of stabilization of the fat emulsion, resulting from various processing methods.

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EFFECT OF INITIAL VITAMIN A STATUS ON SUBSEQUENT RESPONSE OF HOLSTEIN CALVES TO CAROTENE INTAKE FROM ARTIFICIALLY DEHYDRATED ALFALFA[1]

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SUMMARY

Thirty-six male Holstein calves, 89 ± 9 days of age and partially depleted of their vitamin A stores to an average of 8.9 ± 4.0 γ per 100 ml. of plasma, were fed one of three initial carotene intakes, 100, 300, or 900 γ per lb. live weight per day, for a 6-wk. period, and thereafter, one of four final carotene intakes, 20, 60, 180, or 540 γ, for a 12-wk. period. Both plasma carotenoids and vitamin A concentrations were found to respond to current carotene intake, with the more rapid changes observed when the carotene intake was changed from a high to a low intake, as compared with a low or a medium to a high. The initial vitamin A status, as indicated by plasma concentrations at the termination of the initial carotene intake period, influenced the response of plasma and liver vitamin A concentration observed for the final 12-wk. carotene intake period, in that greater carotene intakes were required to obtain a desired response in the case of the lower initial plasma vitamin A concentrations. Based on calculated tocopherol intakes, artificially dehydrated alfalfa resulted in increased plasma and liver concentrations of this vitamin.

Artificially dehydrated alfalfa is widely used in ruminant rations as a source of carotene, pro-vitamin A. The amount of carotene necessary in the ruminant's diet is considerably less after a good pasture season than after a poor pasture season, since high carotene intakes give correspondingly high body stores of vitamin A from which the animal can draw during the subsequent barn-feeding period *(8)*. In the dairy calf, the vitamin A status is dependent upon the dam's previous carotene intake *(7, 13)*, as well as on the carotene content of the feeds fed to the calf *(9, 17)*. To provide information for efficient and optimum utilization of artificially dehydrated alfalfa in calf rations, the present study was undertaken to ascertain quantitatively the contribution of various initial vitamin A stores of the calf to the subsequent utilization of graded levels of carotene intake from artificially dehydrated alfalfa.

EXPERIMENTAL PROCEDURES

Animals and feeding. Thirty-six one-day-old Holstein male calves obtained from various state institution herds during the period August–November, 1955,

Received for publication May 13, 1957.

[1] This study was made in part with grant-in-aid funds provided by the American Dehydrators Association, Kansas City, Mo., the Chas. M. Cox Co., Boston, Mass., and the Big-Y-Foundation, Norwich, Conn.

[2] Present address: U. S. Fishery Products Lab., Ketchikan, Alaska.

[3] Present address: Chas. M. Cox Co., Boston, Mass.

were brought to the research barn and placed in individual tie-stalls. Each calf upon arrival received one 500-mg. oblet of chlortetracycline and 200,000 U.S.P. units of vitamin A from a feeding oil containing 25% lecithin by weight. Thereafter, each calf was raised until the 63rd day of age on a standard limited whole milk, limited dry calf starter, and ad libitum mixed hay regimen. On the 64th day of age, each calf was placed on a vitamin A depletion ration until its blood plasma vitamin A level, determined at successive seven-day periods, decreased to less than 12.0 γ/100 ml. One seven-day period from this time, each calf was assigned to one of the three initial carotene intakes from artificially dehydrated alfalfa leaf meal (obtained from the National Alfalfa Dehydrating and Milling Co., Kansas City, Mo., and containing no additives such as antioxidants, oils, etc.) of 100, 300, or 900 γ/lb live weight/day. After six consecutive weeks of this initial carotene intake period, calves in each initial carotene intake group were subsequently fed for a 12-wk. period one of four final carotene intakes, 20, 60, 180, or 540 γ/lb live weight/day. The first 12 calves to arrive at the research barn, Replicate I, were assigned randomly to one of the three initial and one of the four final carotene intakes. This procedure was repeated for two additional replicates. At the termination of the final carotene intake period, each calf was slaughtered.

At the start of the initial carotene intake period, the calves averaged, with their standard deviations, 89 $\pm$ 9 days of age, 216 $\pm$ 25 lb., and had plasma vitamin A concentrations of 8.9 $\pm$ 4.0 γ per 100 ml. and plasma tocopherol concentrations of 0.115 $\pm$ 0.035 mg. per 100 ml.

Intake of the depletion ration, calculation of the amount of alfalfa to be fed, incorporation of the alfalfa into the depletion ration, and treatment for scours were identical to those reported previously, Rousseau *et al. (17)*, except that the calves were housed in a portion of the barn in which the temperature was not maintained at a minimum of 50° F. and exposure to light was not limited to incandescent artificial light for a 12-hr. period daily. Average minimum and maximum daily temperatures for the final carotene feeding period, with their standard errors were, respectively, 47.9 $\pm$ 0.3° F. and 58.2 $\pm$ 0.3° F. Average light intensity (natural and artificial), measured at a height of 48 in. in the center of each calf stall (in most cases at noon) at successive 28-day periods, was 8.9 $\pm$ 1.3 foot-candles.

Observations and analyses. All feeds fed and refused were weighed daily to the nearest 0.1 lb. Live weights were taken at successive seven-day age periods throughout the experiment, and linear growth measurements, height at withers, heart girth, and circumference of barrel, at the beginning and end of the final carotene intake periods.

Blood samples were obtained by jugular puncture at successive seven-day age periods during the depletion period and at successive 14-day age periods during the initial and final carotene intake periods. Plasma carotenoids and vitamin A were determined by the Kimble procedure *(10)*. Plasma tocopherol was determined by saponification of the plasma by the method of Swick and Baumann *(19)* before extraction with Skellysolve-B and subsequent analysis by the method of Quaife and Biehler *(14)*.

The day before slaughter, at the completion of the final carotene intake period, spinal fluid pressures were recorded, using the procedure of Moore *et al. (12)*. At slaughter the liver was removed, ground in a Hobart food chopper, sampled, and held at 0° F. Liver carotenoids and vitamin A were determined by the Davies method *(6)*. Total tocopherol and lipids were determined by a modification *(4)* of the method of Quaife and Harris *(15)*. The molecular distillates were chromatographed on Floridin (Florex, XXS, 60-90 mesh),[4] rather than employing selective hydrogenation for removal of interfering compounds before the determination of total tocopherols by the Emmerie-Engel reaction *(2)*. Dry matter was determined by drying at 100° C. for 24 hr.

Samples of both the basal ration and artificially dehydrated alfalfa leaf meal were obtained at approximately 4-wk. periods and analyzed for proximate constituents by A.O.A.C. procedures *(1)* (Table 1). The concentration of carotene in the alfalfa was analyzed periodically throughout the course of the experiment, as previously described *(17)*, and the tocopherol content was determined in the same manner as the liver, except that the samples were extracted initially with hot alcohol for 12 hr. rather than for 20 hr. The alfalfa leaf meal contained 277.5 ± 9.5 μg. of carotene per gram and 0.20 ± 0.01 mg. of tocopherol per gram.

TABLE 1

Average chemical composition of feeds

Feed	Dry matter	Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	Carotene
	(%)			*(% dry matter)*			*(mg/lb)*
Mixed hay chopped	92.16 ± 0.54[a]	17.98 ± 0.81	2.17 ± 0.19	29.44 ± 2.32	42.47 ± 2.16	7.94 ± 0.56	22.26 ± 3.85
Calf starter	92.29 ± 0.50	19.70 ± 1.12	3.05 ± 0.22	6.30 ± 0.06	63.44 ± 1.45	7.51 ± 0.22	0.24 ± 0.02
Vitamin A depletion mixture	91.53 ± 0.54	15.93 ± 0.31	2.49 ± 0.14	12.77 ± 0.32	61.87 ± 0.57	6.90 ± 0.12	0.09 ± 0.00
Artificially dehydrated alfalfa leaf meal	93.54 ± 0.42	23.55 ± 0.39	4.47 ± 0.21	19.20 ± 0.51	40.53 ± 0.35	12.25 ± 0.21	125.98 ± 71.95

[a] Standard error of the mean.

Statistical procedures as outlined by Cochran and Cox *(5)* and Snedecor *(18)* were used in the analysis of the data. The data of one calf fed the 900 γ carotene intake during the initial carotene feeding period and 180 γ carotene during the final period were not used in the statistical analyses, because of an extensive and active urogenital infection. Missing values for this calf were calculated according to standard procedures for a randomized block design experiment.

RESULTS AND DISCUSSION

Feed consumption, health, and growth. Feed refusals, days with scours, and the number of treatments with sulfamethazine for scours were infrequent in this study and unaffected by either initial or final carotene intake.

[4] Floridin Co., Tallahasse, Fla.

TABLE 2

Effect of initial carotene intake upon response of plasma carotenoids and vitamin A to subsequent carotene intake from artificially dehydrated alfalfa

Initial carotene intake	Final carotene intake	Plasma carotenoids						Plasma vitamin A		
		Initial carotene intake period		Final carotene intake period				Initial carotene intake period	Final carotene intake period	
		Terminal		Average		Terminal				
		Actual	Log	Actual	Log	Actual	Log	Terminal	Average	Terminal
(γ/lb live weight/day)		*(γ/100 ml.)*								
100	20	70	1.852	21	1.319	20	1.287	23.6	10.3	8.3
	60	52	1.709	37	1.558	45	1.653	17.1	16.6	18.8
	180	52	1.681	93	1.958	111	2.044	15.8	22.0	23.5
	540	42	1.610	179	2.245	220	3.508	18.8	27.1	28.6
300	20	100	2.000	19	1.232	15	1.173	22.0	11.8	8.0
	60	124	2.084	47	1.644	45	1.643	23.9	18.5	15.7
	180	126	2.093	108	1.521	117	2.062	28.4	27.9	28.8
	540	96	1.977	148	2.173	194	2.284	26.3	30.5	29.7
900	20	234	2.364	27	1.363	16	1.195	28.1	15.7	10.4
	60	255	2.382	56	1.705	46	1.672	27.4	21.2	18.2
	180	197	2.295	84	1.911	84	1.892	24.0	24.4	22.6
	540	179	2.243	175	2.231	196	2.287	29.0	30.5	30.7
Standard deviation of a calf		—	0.116	—	0.084	—	0.091	4.0	3.3	3.7

Growth of the calves was inappreciably affected by carotene intake. The average live weight at the beginning of the final carotene intake period was 295 lb., with a standard deviation per calf of 28. Similar values in inches for height at withers, heart girth, and circumference of barrel were, respectively, 38.0 ± 0.9, 43.3 ± 1.9, and 53.0 ± 2.0. The average increases during the 12-wk. final carotene intake period were 191 ± 10 lb. for live weight, 5.3 ± 0.5 in. for height at withers, 8.8 ± 1.1 in. for heart girth, and 10.3 ± 2.1 in. for barrel.

Vitamin A. Plasma carotenoids and vitamin A concentrations at the termination of the initial carotene intake period, as well as the average and terminal values for the final carotene intake period, are given (Table 2), grouped according to initial and final carotene intakes. Values for liver concentrations at the termination of the final carotene intake period are shown (Table 3). Plasma carotenoids and vitamin A concentrations, as affected by time intervals during the final carotene intake period, are given (Figure 1).

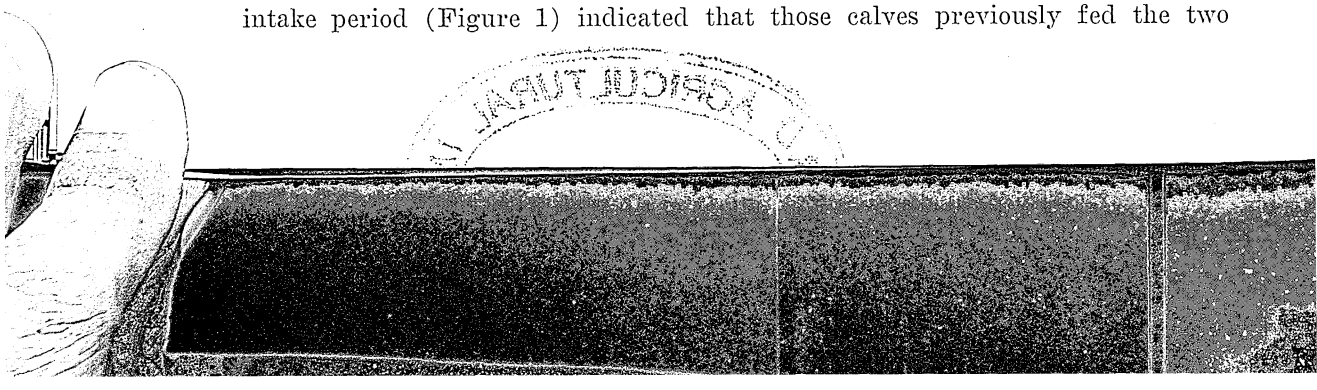
TABLE 3

Effect of initial carotene intake upon response of liver criteria and spinal fluid pressure to subsequent carotene intake from artificially dehydrated alfalfa [a]

Initial carotene intake	Final carotene intake	Liver weight	Liver dry matter	Liver lipids	Liver carotenoids		Liver vitamin A		Spinal fluid pressure
					Actual	Log	Actual	Log	
(γ/lb live wt/day)		*(g.)*	*—(g/100 g.)—*		*——————(γ/100 g.)——————*				*(mm.H_2O)*
100	20	3,858	28.78	3.89	34	1.518	18	1.159	129
	60	3,323	28.54	3.48	59	1.767	194	2.270	104
	180	3,377	27.73	3.55	150	2.173	1,009	3.004	90
	540	3,443	28.62	3.58	259	2.407	2,268	3.356	95
300	20	3,350	28.31	3.77	43	1.635	68	1.719	122
	60	3,404	27.85	3.63	73	1.854	337	2.519	97
	180	3,709	27.80	3.51	183	2.252	1,320	3.070	90
	540	3,513	27.87	3.71	278	2.442	2,360	3.341	97
900	20	3,345	27.97	3.67	39	1.591	83	1.857	118
	60	3,370	28.12	3.56	77	1.876	292	2.356	98
	180	3,233	27.25	3.54	148	2.170	1,144	3.105	90
	540	3,405	27.94	3.71	291	2.463	2,778	3.396	90
Standard deviation of a calf		275	0.68	0.16	—	0.025	—	0.234	14

[a] All values observed at the termination of the 12-wk. final carotene intake period.

It would appear that both plasma carotenoids and vitamin A were responsive to current carotene intake, for changes were observed in these two criteria with a change in carotene intake. As illustrated (Figure 1), more rapid changes were generally associated with the calves previously fed a high intake followed by a low, in contrast to those fed a low intake followed by a high. In addition, the initial vitamin A status, expressed as either plasma carotenoids or plasma vitamin A concentrations at the termination of the initial carotene intake period, measurably affected the response to carotene intake during the final carotene intake period. For example, inspection of the average plasma values by initial and final carotene intake groups plotted against days during the final carotene intake period (Figure 1) indicated that those calves previously fed the two



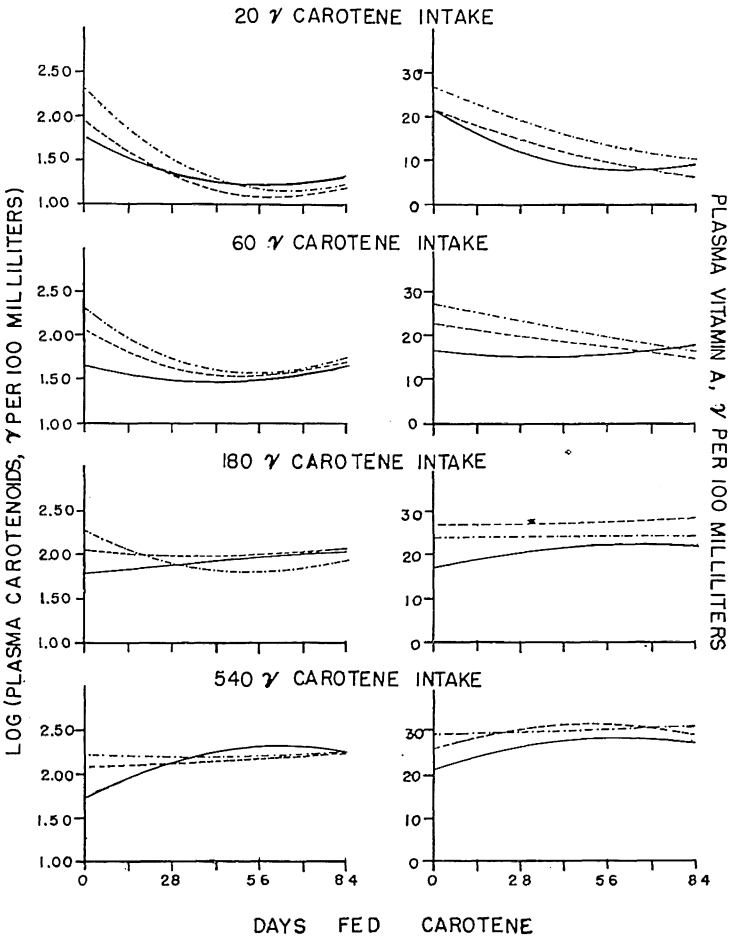


FIG. 1. Effect of initial plasma carotenoid and vitamin A levels (fed previously for a 6-wk. period: ———— = 100 γ carotene/lb live weight/day, – – – – = 300 γ, and – · – · – · – · = 900 γ) upon subsequent response to graded levels of carotene from artificially dehydrated alfalfa.

highest carotene intakes, thus having higher initial plasma concentrations, in general maintained higher average and terminal plasma concentrations during the final carotene intake period, particularly vitamin A, than did those calves previously fed the lowest carotene intake. Similar evidence is shown (Table 3) with respect to liver concentration of carotenoids and of vitamin A.

To express quantitatively the influence of initial vitamin A status upon subsequent response to carotene intake by calves, multiple regressions of response on the logarithm (Base 10) of carotene intake in γ/100 lb. live weight/day, x_1, as well as on the initial vitamin A status, expressed as the plasma vitamin A concentration in γ/100 ml. at the termination of the initial carotene intake period x_2, were derived. For plasma vitamin A concentration in γ/100 ml. at

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the termination of the final carotene intake period, y_{PA}, the relationship was best expressed as follows:

$$y_{PA} = -3.6 + 9.212x_1 - 0.227x_2 + 0.224x_1x_2 \pm 3.3$$

and for log (liver vitamin A in γ/100 g.), at the termination of the final carotene intake period, y_{LA}, the relationship was:

$$y_{LA} = 0.38 + 1.039x_1 - 0.012x_2 + 0.008x_1x_2 \pm 0.27$$

These relationships accounted for 86.7 and 87.1% of the variability about their respective dependent variables, and had applicable limits of prediction between 20 and 540 γ carotene intake for x_1, and between 13 and 33 γ vitamin A/100 ml. of plasma for x_2.

The significance of these regressions can be seen by reference to Figure 2, in which combinations of carotene intake, x_1, and initial plasma vitamin A, x_2, resulting in equal terminal plasma vitamin A concentrations or terminal log (liver vitamin A concentrations), are graphically presented. These contour

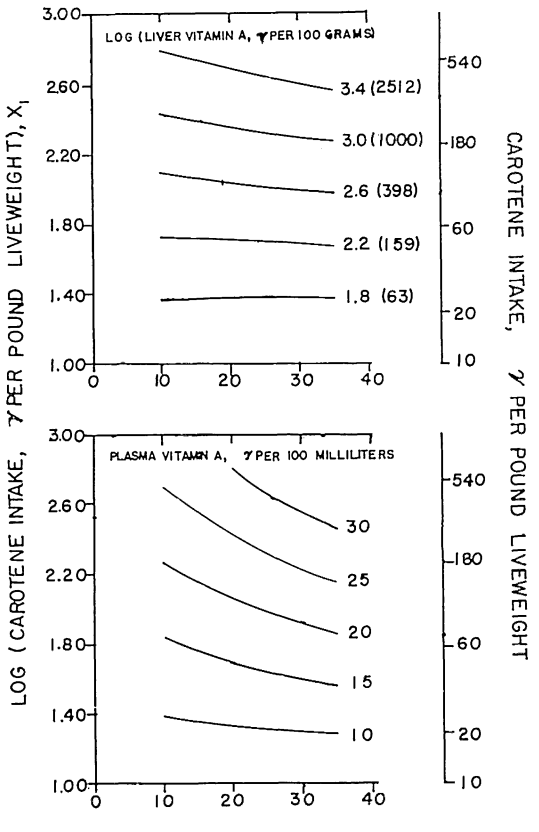


FIG. 2. Combinations of initial plasma vitamin A concentrations and log (carotene intake) from artificially dehydrated alfalfa leaf meal which will result in the indicated responses of plasma vitamin A or log (liver vitamin A), actual liver vitamin A in parentheses, at the termination of a 12-wk. intake period.

lines of response clearly indicate the need for greater carotene intake, to obtain a desired response at the lower initial blood plasma vitamin A concentrations rather than at the higher concentrations. Further, the magnitude of the difference in carotene intake needed between any two initial plasma vitamin A concentrations becomes greater as the desired response is increased from the lower responses to the higher.

Neither liver dry matter nor liver lipids (Table 3) were appreciably affected by the initial or final carotene intakes. Terminal spinal fluid pressures of the calves fed the 20 γ final carotene intake were greater than those of the calves fed the three other final carotene intakes. The magnitude of the spinal fluid pressures in the 20 γ carotene intake grouping was inversely related to the initial carotene intake, i.e., calves fed the 900 γ carotene intake initially had lower pressures than those fed 300 γ initially, and those fed 300 γ lower values than those fed 100 γ.

In terms of application of these statistics, two generalizations seem apparent: First, the vitamin A status of the calf, as indicated by the current plasma vitamin A concentration, is responsive to the current carotene intake (Figure 1). More rapid changes were observed when calves were fed a low intake following a high, than when they were fed a high intake after a low or medium intake. Secondly, the previous vitamin A status of the calf affects the carotene intake required for a given response, such as those studied herein (Figure 2). It needs to be recognized in the second generalization that the regressions given are point-in-time estimates, i.e., observations taken after 12 wk. of the of the indicated carotene intakes. Had the final carotene intake period been twice the duration of that studied herein, the effect on the terminal plasma and liver concentrations of initial vitamin A status would possibly have been nil, since it would have been anticipated that plasma vitamin A concentrations (thus, liver vitamin A also) for calves fed a particular final carotene intake would have approached a common level. However, such does not negate the influence of initial vitamin A status, for those calves with the higher initial plasma vitamin A concentrations would have taken a longer period of time to reach the same plasma (Figure 1) or liver vitamin A concentrations as those with the lower initial plasma vitamin A concentrations.

Tocopherol. Since artificially dehydrated alfalfa is an excellent source of tocopherol *(3, 11)*, as well as of carotene, it was of importance to determine the response of plasma and liver tocopherol concentrations to tocopherol intake from the alfalfa added to the depletion ration. Such data for the final carotene intake period are shown (Table 4). The tocopherol intakes in mg/lb live weight/day were calculated from the grams of alfalfa leaf meal fed per pound of live weight/day multiplied by the average tocopherol concentration of the alfalfa leaf meal in mg/g.

Accepting the previously suggested relationships between plasma and liver tocopherol concentrations and tocopherol intake *(16)*, it was found that the average plasma tocopherol concentration during the final carotene intake period, adjusted for the terminal values of the initial carotene intake period, increased

TABLE 4

Response of plasma and liver concentrations of tocopherol to added tocopherol from artificially dehydrated alfalfa

	Calculated tocopherol added to the depletion ration from alfalfa in mg/lb live weight/day				Standard deviation of a calf
	0.014	0.043	0.130	0.389	
Plasma tocopherol	*(mg/100 ml.)*				
Terminal value of initial carotene intake period	0.172	0.153	0.140	0.156	0.038
Av. value of final carotene intake period	0.146	0.151	0.161	0.197	0.022
Av. value of final carotene intake period adjusted for terminal value of initial carotene intake period	0.130	0.151	0.163	0.197	0.021
Liver tocopherol	*(mg/100 g.)*				
Terminal value of final carotene intake period					
Actual	0.236	0.276	0.281	0.365	—
Log [a]	0.364	0.437	0.437	0.554	0.092

[a] Log [(mg/100 g.) (10)].

0.044 mg/100 ml. per log (tocopherol intake from alfalfa in mg/lb live weight/day times 100) with a standard error of 0.002. Log (liver tocopherol times ten) increased 0.118 mg/100 g. per log (tocopherol intake from alfalfa in mg/lb live weight/day times 100) with a standard error of 0.021. These linear regression coefficients accounted for 96.0% of the variability about plasma tocopherol and for 87.5% of that about log (liver tocopherol) concentrations. Though admittedly based on calculated rather than determined tocopherol intakes, these data indicated that artificially dehydrated alfalfa contributes to the tocopherol status of the calf, as indicated by increases in concentrations of this vitamin in the plasma and liver.

ACKNOWLEDGMENTS

The authors are most grateful to B. A. Donohue, R. Landeck, and T. Watts for the care and feeding of the calves; to E. L. Bacon, Mrs. Elaine Trantum, and Mrs. Mae Miller for technical assistance, and to H. J. Fisher, Connecticut Agricultural Experiment Station, New Haven, for proximate analyses of the feedstuffs. The Nopco Chemical Co., Harrison, N. J., through the courtesy of M. Hochberg, supplied the vitamin A feeding oil, and the American Cyanamid Co., Pearl River, N. Y., through the courtesy of R. F. Elliott, provided the chlortetracycline and the sulfamethazine for the prevention and treatment of scours. We wish to express our thanks to D. L. Rucker, Sales Dept., National Alfalfa Dehydrating and Milling Co., Kansas City, Mo., for the many arrangements necessitated by purchase and shipping of the artificially dehydrated alfalfa leaf meal.

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MOTILITY OF BOVINE SPERMATOZOA STORED AT 5° C. WHEN EXTENDED IN MIXTURES OF YOLK-CITRATE, YOLK-GLYCINE, WHOLE MILK, SKIMMILK, AND GLYCEROL[1, 2]

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SUMMARY

Four semen samples were collected from each of three bulls. Each semen sample was divided into a glycerolated (7.5%) and a nonglycerolated group. Each group was extended 1:24 into ten different extenders at an initial temperature of 32° C. The extenders were egg yolk–citrate (YC), whole milk (WM), skimmilk (SM), and egg yolk–glycine (YG), and their combinations were made up in equal proportions. Progressive motility rated on a 0-10 scale (10=100%) was used to estimate the desirability of the extenders during storage at 5° C. YC, WM, SM, and YG were, on the average, lower in motility after extension at Day 3 and Day 7 of storage ($P < 0.01$) than their combinations. Both combinations, WMYG and SMYG, showed higher average motility on Day 7 ($P < 0.01$) than did WMYC, SMYC, WMSM, or YCYG. YCYG was superior ($P < 0.01$) to either YC or YG alone. WM, SM, and WMYC had higher motility after extension ($P < 0.05$) than the same extenders containing glycerol. No other comparisons involving glycerol showed statistically significant differences. Under the conditions of this study, 2.9% glycine enhanced the ability of sperm to maintain progressive motility under storage at 5° C. when used with WM, SM, or YC.

For routine artificial insemination, egg yolk–citrate *(18)*, heated homogenized milk *(2, 12, 22)*, and heated skimmilk *(2, 22)* are the extenders most widely used *(4)* for bull semen. Fertility of semen stored in the liquid state in these extenders is generally unsatisfactory by the fourth day after collection. Attempts to improve livability of semen in yolk-citrate extenders include varying the ratio of ingredients *(4, 11)*, using dialyzed yolk *(23)* and whole egg *(3)*. Johnson *et al. (9)* concluded that no improvement in sperm livability can be expected by alteration of the pH, osmotic pressure, or buffering capacity of milk extender.

Additives such as glycine *(6, 12, 18, 24)*, glycerol *(14, 26)*, and sodium bicarbonate *(11, 16, 17, 25)* have been more recently used. Limited results are available from trials combining basic extenders. In one study *(4)*, combinations of 15% yolk, 15% citrate, and 70% skimmilk showed a 70% nonreturn rate at 60-90 days. In another trial *(4)*, in which 5% yolk was added to milk for semen used the second day after collection, the 60-90 day nonreturns were increased 6%.

This study was undertaken to determine changes in sperm motility at 5° C. as influenced by extension in mixtures of yolk-citrate (YC), whole milk (WM), skimmilk (SM), yolk-glycine (YG), and glycerol.

Received for publication November 4, 1957.

[1] Scientific Paper No. 1669, Washington Agricultural Experiment Station, Pullman. Project 1107.

[2] The data are from a thesis presented by the senior author to the graduate faculty of the State College of Washington in partial fulfillment of the requirements for the Ph.D. degree.

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MATERIALS AND METHODS

Four semen samples were collected from each of three bulls from December 31, 1955, to March 23, 1956. Immediately after collection, the semen samples were put in a 32° C. water bath. Because of the distance between the bull barn and the laboratory, about 15 min. elapsed between collection and the start of laboratory processing.

Each semen sample was divided into glycerolated and nonglycerolated (control) groups. The glycerolated group of extenders contained 7.5% glycerol when the semen was added. This is a departure from the usual method of step-wise glycerolation after the partially extended semen has cooled. Each group was extended 1:24 into ten different extenders containing 1,000 μg. streptomycin/ml of extended semen. The extenders used were: A. YC; B. ½ YC + ½ WM; C. ½ YC + ½ SM; D. ½ YC + ½ YG; E. WM; F. ½ WM + ½ SM; G. ½ WM + ½ YG; H. SM; I. ½ SM + ½ YG, and J. YG. The yolk-citrate was composed of ½ egg yolk and ½ sodium citrate (2.9% solution). The yolk-glycine was composed of ½ egg yolk and ½ glycine (2.9% solution). Practical-grade glycine was used and has given better results than the purer, ammonia-free compounds. The whloe milk and skimmilk extenders were prepared by the method of Thacker and Almquist *(22)*, except that the milk was heated to boiling and then cooled rapidly.

Progressive motility was rated on a 0-10 scale (10 = 100%). After initial examination, the extended semen was stored at 5° C. and observed daily until motility ceased. All samples were randomized and then rated by two persons (using low-power microscopy), with a third person preparing slides maintained at 37° C. *(8)*. After the seventh day of storage only the senior author examined the samples, until they rated zero motility. Analysis of variance using individual degrees of freedom was as outlined in Snedecor *(21)*. The analysis was made using the motility values as determined by each of two raters at the start of storage (initial) and for Day 3 and Day 7 of storage. References to statistical significance in the text are restricted to the storage times mentioned above.

RESULTS AND DISCUSSION

As shown (Table 1), motility of sperm in any of the basic extenders, YC, WM, SM, or YG (including glycerol treatment) was, on the average, inferior ($P < 0.01$) as compared with combinations of these extenders. YG was superior to YC ($P < 0.01$) initially but not at Day 3 or Day 7. WM or SM, used in combination with YC, showed higher average motility on Day 7 ($P < 0.01$) as compared individually with WMYC, SMYC, WMSM, or YCYG.

Comparisons involving addition of 7.5% glycerol were variable from one extender to the next (Table 1). The greatest differences in motility were noted initially. Nonglycerolated WM, SM, and WMYC had significantly higher motility initially ($P < 0.05$) than glycerolated WM, SM, or WMYC. All other comparisons involving glycerol were not significant. Sperm extended in glycerolated YG or SM showed higher average motility on Day 7 than YG or SM

TABLE 1

Effect of various combinations of semen extenders upon percentage of motile sperm during storage at 5° C.

Day	A YC[a]	B ½ YC ½ WM[a]	C ½ YC ½ SM[a]	D ½ YC ½ YG[a]	E WM	F ½ WM ½ SM	G ½ WM ½ YG	H SM	I ½ SM ½ YG	J YG	Average
					No glycerol						
Initial	53	68	64	56	65	60	65	60	65	61	61
1	54	65	61	53	62	56	61	50	60	55	57
2	36	52	52	45	50	48	54	42	56	38	47
3	34	52	52	48	50	56	56	44	58	38	48
4	28	54	52	47	48	46	52	35	55	36	45
5	26	48	48	44	47	42	48	36	51	28	42
6	22	36	38	36	40	36	50	26	48	22	36
7	28	35	31	40	40	34	52	20	46	22	35
14	4	8	8	4	5	1	19	0	26	1	10
Average	32	46	45	41	45	42	50	35	52	33	42
					Addition of 7.5% glycerol						
Initial	48	58	58	55	55	56	61	48	56	58	56
1	46	56	46	53	51	45	57	42	56	54	51
2	35	48	46	42	43	43	49	36	46	42	42
3	35	48	46	44	44	48	51	39	50	38	44
4	28	42	42	38	38	40	44	36	50	35	40
5	26	38	36	38	38	40	45	31	50	36	38
6	16	35	26	32	32	36	40	34	40	28	32
7	19	34	32	34	35	38	44	30	46	30	34
14	0	1	2	4	8	2	13	0	14	1	6
Average	28	40	37	38	38	38	45	33	45	36	38

[a] YC=Yolk citrate, WM=whole milk, SM=skimmilk, and YG=yolk glycine.

(not significant). Motility was not depressed at Day 7 in glycerolated SMYC, WMSM, or SMYG. Glycerol appeared least desirable in YC.

WM or SM alone and in combination with YC or YG did not visibly settle out during storage. WMYG appeared to be the most homogeneous mixture. YC and YG exhibited considerable sedimentation by Day 3. It is doubtful that this affected the results, because Kampschmidt *et al. (10)* presented evidence that rapid sedimentation during storage of semen extended in yolk-citrate had no effect on survival.

Further observations using yolk glycine and whole milk. Seven semen samples from two bulls were extended in ¼ WM + ¾ YG, ½ WM + ½ YG, and ¾ WM + ¼ YG, with and without glycerol. Motility was similar in all combinations (35-40%) at Day 7 of storage at 5° C. At Day 21 the nonglycerolated extended semen averaged 19% progressively motile sperm and the glycerolated averaged less than 10%. The extenders containing ¾ YG (glycerolated and nonglycerolated) showed slightly lower average motility at all periods of observation during storage. However, one sample out of the ¾ YG group was rated as 20% in progressive motility after 63 days of storage and still exhibited some motility at 70 days. Roy and Bishop *(18)* reported one semen sample extended in glycine-fructose-egg yolk which appeared satisfactory for insemination after 50 days and contained motile sperm for 65 days.

General discussion. Flipse and Almquist *(5, 6)* reported a longer survival for spermatozoa in skimmilk-glycine than in skimmilk. Skimmilk-glycine was only slightly superior to skimmilk in supporting motility during 1 wk. of semen storage, but during the 2nd wk. of storage skimmilk-glycine clearly was better *(6)*. In the present study, glycine was not separately added to milk, but the beneficial effect of combining yolk and glycine with milk was shown. Flipse and Almquist *(5, 6)* found that egg yolk–glycine supported motility better than skimmilk-glycine during prolonged storage of semen. Results of the present trials indicate that yolk-glycine and skimmilk are similar but are inferior to whole milk.

Tyler and Rothschild *(24)* have suggested that the beneficial effect of glycine on sperm survival is the result of chelation of heavy metals. Roy and Bishop *(18)* believe that, in addition, glycine must have other beneficial effects, perhaps involving its low electrolyte content or its important role as a precursor for such substances as creatine and adenosine. Glycine *(7)* has excellent buffering capacity within a pH range of 5-7, which is satisfactory for stored extended semen. Buffering capacity and pH are important considerations when developing extenders for semen.

Smith *et al. (20)* have shown that maintenance of near constant osmotic pressure also is essential. They found that media containing glucose aid in the maintenance of osmotic pressure within narrow limits and were associated with maximum sperm livability during a ten-day storage period. Lardy and Phillips *(13)* have shown that energy for motility is obtained through glycolysis. O'Dell *et al. (15)* have shown that glycerol-1-C^{14} can be metabolized by spermatozoa and that this utilization was greater when seminal plasma was not present. Albright

et al. (1) have shown that sperm freed from seminal plasma by washing twice with 2.9% sodium citrate can use energy sources in milk to maintain motility during incubation at 37° C.

From the results (Table 1), it appears that the extenders providing the greater variety of constituents showed the highest average motility after seven days of storage at 5° C. At seven days, WMYG showed the highest average motility, followed closely by SMYG and glycerolated SMYG and WMYG. Extenders containing glycerol were, in general, lower in motility immediately after extension, but declined at a lesser rate during storage than the same extender minus glycerol.

Glycerolated (7.5%) SM was superior to SM alone and glycerolated (7.5%) YG was superior to YG alone after seven days of storage. However, these were significantly lower ($P < 0.01$) after seven days of storage than SMYG with and without 7.5% glycerol. Flipse and Almquist did not add 2.5% and 5.0% glycerol separately to skimmilk and these levels in yolk-glycine were not beneficial *(6)*. However, addition of 5.0% glycerol was beneficial during prolonged storage of semen extended in reconstituted nonfat dry milk solids containing 0.5 *M* glycine (1:1).

From the present study, it appears that SMYG has augmenting properties which equal SMYG + glycerol and exceed SM + glycerol or YG + glycerol. Likewise, YG in combination with WM or YC was superior to WMYC or YC. Under the conditions of this study, glycine has a property or properties which augment motility retention in semen extended in YC, WM, or SM during 5° C. storage.

ACKNOWLEDGMENT

The technical assistance of Mary McGreevy and Jennie Rice is gratefully acknowledged.

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FLOW DIALYSIS AS A MEANS OF PRESERVING BOVINE SEMEN AT ROOM TEMPERATURE[1]

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SUMMARY

A flow dialysis technique has been developed and used to prolong the life of bull sperm at room temperatures. Samples of undiluted semen in dialysis sacs were suspended in constantly flowing media of egg yolk–citrate, of milk, and of blood products. Undiluted semen controls in test tubes at room temperature lived for about 24 hr.; whereas, portions of the same samples undiluted in dialysis sacs survived for a week, with a yolk-citrate media constantly passing the sacs. Survival was better with the yolk-citrate mixture in the flow dialysis system than with any of the other media tried. Sperm life was supported better at room temperature than at 5° C. in the flow dialysis system. Bubbling O_2 through yolk-citrate media decreased the survival time of sperm compared to no gassing, whereas N_2 improved survival. Results from gassing with CO_2 varied from much improvement in survival at times to reduced survival in others. Further studies on the effects of CO_2 on bull sperm are in progress.

The in vitro preservation of spermatozoa has been accomplished in the past largely by storing diluted semen at reduced temperatures. Yet, the long survival (for from 60 to 70 days) and maintenance of fertility (for from 30 to 40 days) of spermatozoa, in the epididymides ligated from the testes of males of several species, have been known for from 20 to 30 yr. *(4, 5, 7, 10)*. Thus, high temperature alone would not seem to be the limiting factor in maintaining viable, fertile sperm for periods of several days. The epididymis, with its constantly changing blood supply, undoubtedly affords a ready source of nutrients and a rapid means of removing waste products. Duplication of these conditions might provide a means of storing semen without the use of refrigeration.

In the present study, attempts have been made to maintain bull sperm at room temperature by using modifications of a membrane technique which bacteriologists, biochemists, and physiologists have used many years *(1, 3)*. A constantly changing environment for the sperm has been supplied by using flow dialysis. Since the start of the studies reported here, a membrane technique has been used by others to study spermatozoa *(2, 9)*. However, a preliminary report of the investigations discussed here seems to be the first to indicate prolongation of sperm survival at room temperatures through the use of a dialysis technique *(8)*. In this study, sperm survival was compared in a flow dialysis system with survival in test tubes at room temperature and at 5° C., using several dialysis fluids, various rates of fluid flow, and several gaseous environments.

METHODS

Provisions for renewing the substrates available to sperm and eliminating waste products were made by arranging a flow dialysis (FD) setup. The system

Received for publication November 6, 1957.

[1] The data presented in this paper were taken from a thesis submitted by the junior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the Master's degree.

consisted of a reservoir for a constant supply of dialysis fluid, a connecting tube to one side-arm of a U-tube, a double side-arm U-tube in which a dialysis sac containing semen was suspended, a connecting tube with a needle valve, and a drainage receptacle (see Figure 1).

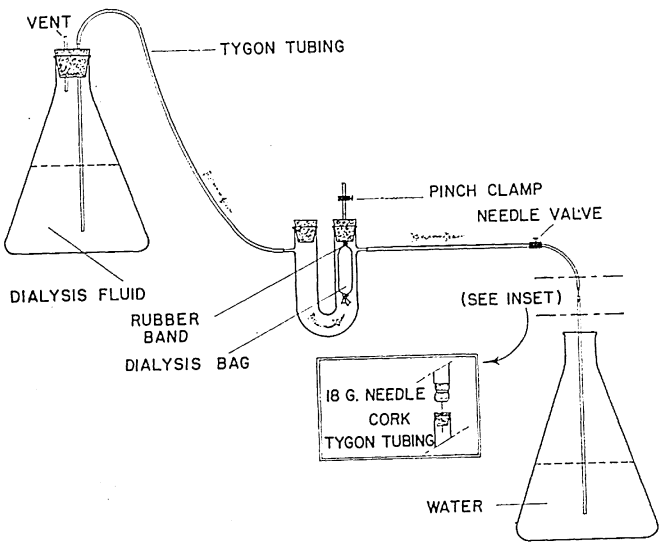


FIG. 1. Flow dialysis system with supply flask, U-tube, and over-flow receptacle. Flow by siphon regulated by needle valve.

One-half milliliter of fresh undiluted bull semen was placed in a previously softened Visking cellulose casing (10 by 35 mm.) which was attached to a small-bore glass tubing inserted through a rubber stopper that would fit the U-tube (see Figure 2). A piece of plastic tubing and a pinch clamp were used to close the opening of the glass tube leading to the casing. Through this opening, samples of semen were removed by syringe and needle, periodically during the experiments, for a microscopic check for sperm survival. The clamp also prevented the casing from collapsing due to the pressure of the dialysis fluid after the system was assembled and the flow was started.

A number of means of regulating the rate of flow of the dialysis fluid past the membrane were tried. The use of a siphon with gravity flow regulated by a needle valve between the U-tube and the waste receptacle proved more satisfactory than using pressure, vacuum, or fluid displacement as a means of regulating flow. The rate of flow in each experiment was regulated to 250 ml. per 24-hr. period, unless otherwise indicated.

Gassing was accomplished by slowly bubbling the gases through the fluid in the supply flask. Too rapid gassing, except with clear diluents, caused excessive foaming. The rate of gassing was regulated with a flow meter.

Sperm survival was judged on the basis of the percentage and rate of sperm motility found when a small drop of semen from the dialysis sac was placed on

a microscope slide, diluted with saline, and warmed to body temperature. The activity of the sperm before removal from the dialysis sac was not determined.

RESULTS

Experiments were carried out to compare the flow dialysis system with dilution and storage in test tubes (the conventional system now widely used in the field) as a means of preserving bovine semen. The effects on sperm survival of yolk and milk extenders and blood-derived dialysis fluids, of temperatures of 5 and 26° C., of several gases, and of three rates of flow of dialysis fluid, were among the items tested.

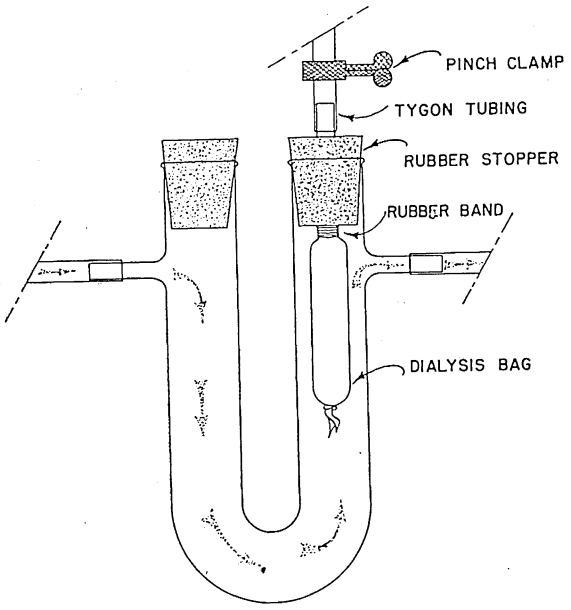


FIG. 2. U-tube assembly used in flow dialysis system.

Survival of sperm at 5 and 26° C. using yolk, milk, and blood-derived media. Each of eight samples of freshly collected bull semen (averaging 51% motile sperm and 1,130 × 10^6 sperm per ml.) was divided into 14 portions. Two portions were left undiluted and were placed in small test tubes: these served as one set of controls (S). Six portions of semen were diluted at a rate of one part semen to ten parts extender and placed in test tubes [diluted controls designated as (C)]. The extenders used were A) YC, 25% egg yolk and 75% of a 2.9% solution of sodium citrate dihydrate, B) YCM, a mixture of 30% of YC and 70% unheated pasteurized skimmilk, and (C) M, pasteurized skimmilk that had been heated to from 92 to 95° C. for 10 min. Sulfanilamide was added to all extenders at a concentration of 3 g. per liter. Other additives included one million I.U. of penicillin and 2 g. of dihydrostreptomycin sulfate per liter of extender. The last six portions of semen (0.5 ml. each) were placed in each of six flow dialysis systems. The extenders used in the second set of controls served

as the dialysis fluids in these systems. One-half the prepared portions was held at 26° C., the other half at 5° C., after slow cooling to prevent shock owing to the cold.

The results of this experiment are shown (Figures 3 and 4). From Figure 3 it can be seen that sperm survival was best at 26° C. under the conditions in the flow dialysis systems, with those with yolk-citrate as the dialysis fluid outliving all others. The diluted control samples in test tubes at this temperature were

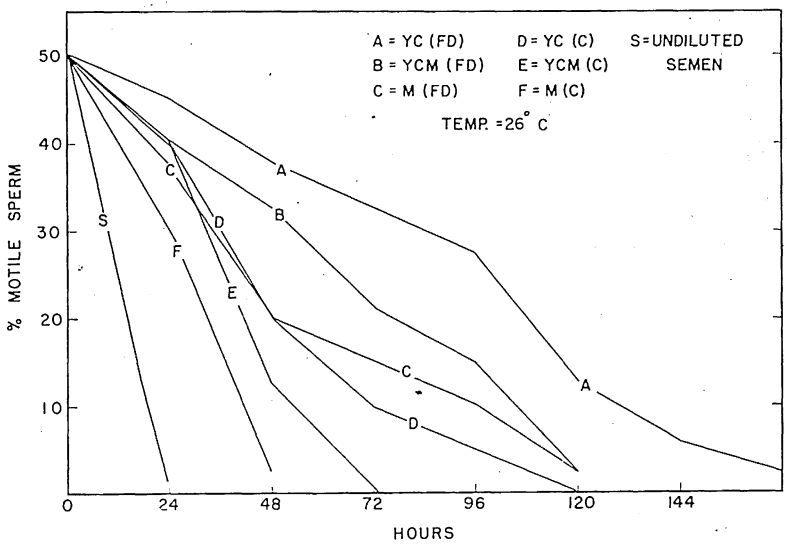


FIG. 3. Comparative sperm survival in the flow dialysis system (FD), in test tube controls (C), and in undiluted semen (S), using three media at 26° C. (Means of eight trials.)

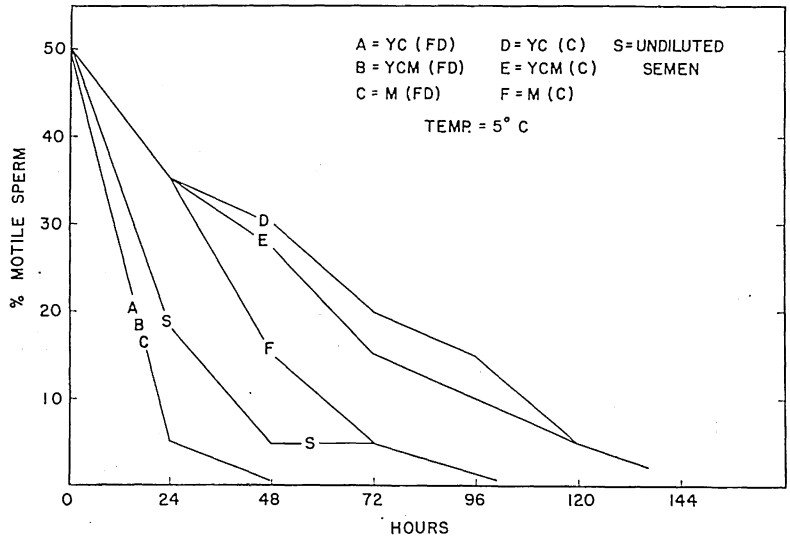


FIG. 4. Comparative sperm survival in the flow dialysis system (FD), in test tube controls (C), and in undiluted semen (S), using three media at 5° C. (Means of eight trials.)

dead from two to five days sooner than those in the FD systems. The undiluted control samples in test tubes at this temperature were dead in 24 hr. At 5° C. (see Figure 4), the diluted control samples outlived those in the FD systems by from two to four days. Apparently, the bull sperm were harmed even by slow cooling if egg or milk was not in contact with the cells. The best over-all survival, when both temperatures were compared, was obtained by flow dialysis at 26° C., with survival lasting a week in some instances.

In a separate experiment involving four samples of semen, to compare sperm survival in blood-derived preparations (defibrinated whole blood, blood serum, and heated blood serum) and yolk-citrate at 26° C., none of the blood preparations as dialysis fluids was as effective in supporting sperm survival as was the yolk-citrate. However, all maintained sperm much longer in the FD systems than either they or yolk-citrate did as direct diluents.

The effect of O_2, N_2, and CO_2 on sperm survival at 26° C. in FD systems. A number of reports in the literature suggest the harmful effects of oxygen and the beneficial effects of anaerobic conditions on the survival of spermatozoa *(6)*. Accordingly, aerobic and anaerobic conditions were produced by the constant gassing of the yolk-citrate dialysis fluid (same as previously described) and a dialysis fluid consisting of 0.9% solution of NaCl with oxygen, nitrogen, or carbon dioxide. Each gas was bubbled through the dialysis fluid in the supply flask at a rate of 15 ml. per minute for 2 hr. before starting the experiment. The same rate of gassing was continued throughout the experiment. Seven semen samples with an average initial percentage of motile sperm of 63% and from 1,641 to 2,203 $\times 10^6$ sperm per milliliter were split and used in this experiment. The percentage of motile sperm and the pH of the dialysis fluids were determined initially and every 12 hr. thereafter. No attempt was made to control pH.

The average percentage of sperm found to be motile upon sampling, diluting with 0.9% NaCl solution, and warming to 37° C. under each of the gases is shown graphically (Figure 5). For the first 60 hr. there was little difference in sperm survival as a result of gassing with N_2, CO_2, or no gassing. From 60 to 156 hr. the samples under N_2 survived somewhat better than the other two. Oxygen depressed sperm survival much below the level observed with no gassing. With all the unbuffered saline systems without gassing, as well as with all the gases, pH dropped below 6.0 and the sperm died rapidly. The pH was maintained at about 6.7 to 6.8 in all the treatments where egg yolk–citrate was present, except in the presence of CO_2; there, the pH remained at about 6.1 to 6.2 throughout the experiment.

The response to CO_2 was extremely variable. In some cases CO_2 appeared to be toxic and in others it was stimulatory to the percentage and rate of sperm motility. At one time or another during the experiment, in six out of the seven trials, sperm motility appeared better preserved in the presence of CO_2 than it did with any of the other treatments. However, these occurred at irregular intervals and were frequently followed by poorer sperm survival; thus, the average survival under the influence of CO_2 was no better than under nitrogen or without gassing. Supplying CO_2 to one sample after much of the motility was lost

with no gassing, brought about a phenomenal recovery both in the percentage of moving sperm and in the rate of their progression.

Effect of rate of passage of the dialysis fluid on sperm survival at 26° C. Since the rate of flow would affect the exchange of nutrients and waste products in a flow dialysis system, sperm survival might be affected by the rate of passage of the dialysis fluid. Rates of flow of 5, 10, and 25 ml. per hour with the previously described yolk-citrate dialysis fluid were tested for their effect on sperm survival. Sulfanilamide, penicillin, and streptomycin were again added. Four ejaculates

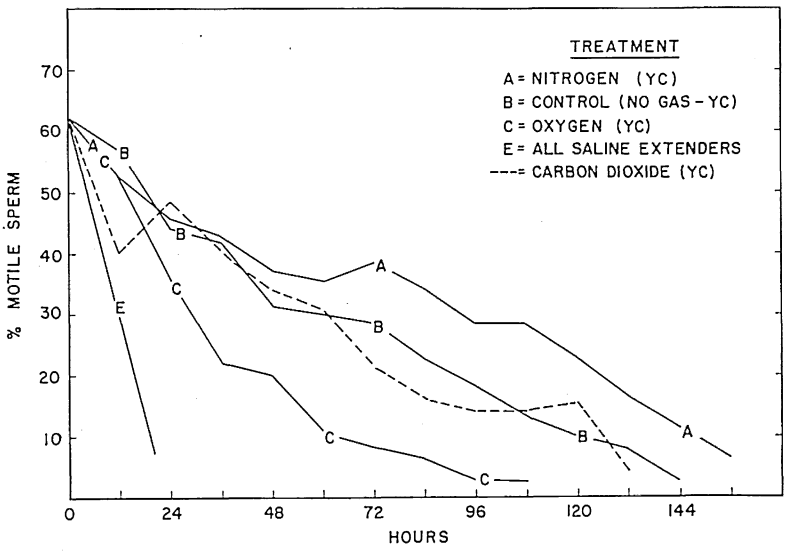


FIG. 5. Comparative sperm survival in the flow dialysis system with yolk-citrate or saline media and various gaseous environments. (Means of seven trials.)

containing from 603 to 1,440 $\times 10^6$ sperm per milliliter and an average of 63% motile sperm were split so that each rate of flow was tested on a portion of each sample.

There was little difference in the survival of sperm at the two slower perfusion rates. However, the 25 ml. per hour level supported motility about one day longer than the two slower rates of flow. The faster rate of flow was easier to adjust and to control than the slowest rate.

DISCUSSION

The flow dialysis system developed and tested in these investigations has shown that the life of bovine spermatozoa in vitro at above-refrigeration temperatures can be greatly extended by removing waste products and supplying nutrients. With the common diluents of bull semen (egg yolk–citrate and milk) as dialysis fluids, and undiluted semen in the dialysis sac, sperm survival was frequently maintained for as long as seven days at room temperature (26° C.). This is in extreme contrast to undiluted semen in test tubes at the same temperature, where

survival seldom lasted longer than 24 hr. Thus, as was hypothesized, temperature alone was not the limiting factor in sperm survival in undiluted semen. In fact, the FD system at 5° C. was not as effective in prolonging sperm survival as it was at 26° C. Even with slow cooling, cold shock may have occurred, since the extenders were not in direct contact with the sperm cells.

Dialysis fluids of bovine blood and serum were not as effective in prolonging sperm survival in the FD system as were the conventional bovine semen extenders. However, sperm survival was much longer when the blood products were used in the FD system than it was when the blood was mixed directly with the semen and stored in test tubes. This is in agreement with the earlier studies that have been cited *(6)*.

The effects of oxygen and nitrogen were much as expected from the previous knowledge of the harmful action of aerobic conditions and the beneficial effects of anaerobic conditions. The variability of the effects of carbon dioxide make results with this gas difficult to interpret. In some cases, CO_2 greatly improved and supported sperm survival; in others, sperm life was shortened. As a result, the over-all average motility under CO_2 was about the same as with no gassing. It could be that CO_2 was beneficial when certain other conditions were properly maintained, but the CO_2 became harmful or ineffective with the changes that occurred during storage. However, there was no doubt that CO_2 was beneficial to the maintenance of sperm survival at room temperature under the conditions which existed in some samples. Further investigations of the effects of CO_2 on bull sperm are under way.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the members of the Illinois Dairy Breeding Federation for the financial aid given in support of this research and to W. O. Nelson for his suggestions on the use of the flow dialysis technique.

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LACTATION STUDIES. I. EFFECTS UPON MILK EJECTION IN THE BOVINE OF VARIOUS INJECTION TREATMENTS USING OXYTOCIN AND RELAXIN [1]

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SUMMARY

A cow became unable, in most instances, to eject milk normally. Oxytocin was administered to this cow intravenously, to study milk ejection. Six hundredths I. U. oxytocin was the least amount that caused partial milk ejection, whereas 0.12 to 0.25 I. U. caused normal ejection. Amounts up to ten units were increasingly more effective. Small amounts of oxytocin were more effective in ejecting milk when injected rapidly rather than slowly. However, there was little difference resulting from two 0.25 I. U. injections within a few minutes, and one 0.5 I. U. Complementary milk increased after a second injection of oxytocin, but decreased in per cent of total milk, and the fat per cent was unchanged as the time between injections increased from one to two milking intervals. Milk from the cistern of the udder increased in volume before milk ejection at milkings subsequent to oxytocin treatment, and decreased in fat as the time extended from one to two milkings and as the amount of oxytocin decreased. More complementary milk, higher in fat, was collected from certain amounts of oxytocin, divided into two injections given at one milking, if the most effective injection was given last. Relaxin was tested for milk ejection properties, and from 750 to 1,800 G.P.U. injected intravenously into three cows on two occasions did not cause noticeable milk ejection.

The early work of Gaines *(5)* introduced the concept that milk ejection was distinct from milk secretion and implicated the posterior pituitary gland and the application of stimuli to the teats with the process of milk ejection. Later, Ely and Petersen *(4)* provided information on the nervous pathways in the milk let-down reflex act, and Petersen and Ludwick *(8)* suggested that milk ejection was brought about by the action of oxytocin released from the posterior pituitary gland as a result of preparation for milking.

Our knowledge of the concentration of oxytocin in the blood that brings about ejections of milk in animals is not extensive. Whittlestone *(12)*, working with sows, has estimated that a concentration of exogenous oxytocin of one part in 10^{10} parts of blood was effective in bringing about a definite ejection response. This level in the blood corresponded to the injection of 0.05 I.U. per injection into a 300- to 400-lb. sow. A response to 0.05 I.U. was elicited only when all of the oxytocin was injected within two seconds or less *(13)*. Previously, Whittlestone *(10)* had reported that as little as 0.025 I.U. Pitocin caused a milk ejection response in a mature sow. The minimum effective quantity of oxytocin to eject milk from rabbits was found by Cross and Harris *(1)* to be 0.005 I.U. per injection. Later, Cross and Van Dyke *(2)* revised their estimate of the minimum effective amount down to between 0.001 and 0.0001 I.U. per injection. Donker

Received for publication June 15, 1956.

[1] Journal Series Paper No. 23 of the Georgia Agricultural Experiment Station, Athens.

[2] This work was done while the author was a member of the Dairy Department, University of Georgia, Athens.

et al. (3) found that complementary milk was effectively removed in certain cows with the aid of 1.5 to 3.0 I.U. oxytocin.

Shaffhausen *et al. (9)* reported that intravenously administered relaxin caused milk ejection in sheep.

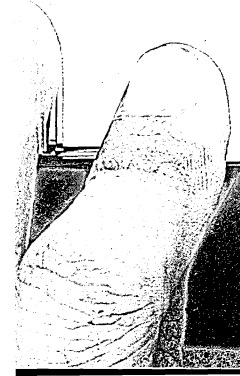
The data in this paper present information on the minimum amounts of oxytocin found effective in bringing about ejections of milk from a cow which had developed a refractoriness to the normal stimuli causing milk ejection. Also, the effect of rate of injection and sequence of injections of oxytocin upon the ejection response was studied and, in a limited way, the effect of relaxin in causing milk ejection was compared with that of oxytocin in the bovine.

EXPERIMENTAL PROCEDURES

Most of the data reported here were obtained from a 5-yr.-old grade Holstein-Jersey cow during 3½ mo. This cow was observed to have become refractory, in most instances, to the stimuli that normally evoked milk ejection; hence, it presented a singular opportunity to study milk ejection. Milk was removed only partially from this cow by the normal milking act (preceded by the usually effective stimuli). This milk, obtained by normal means, will be termed cistern milk. In order to obtain normal amounts of milk, it was necessary to eject the milk with the aid of intravenous injections of oxytocin (complementary milk). The least amount of oxytocin that would eject milk from this animal was ascertained by injecting successively smaller amounts, at consecutive regular milking times, until no response was noted. It was first ascertained whether the animal would respond to the stimuli employed in the routine preparation for milking. On widely separated occasions, the cow responded by ejecting milk more or less normally. At those times, experimental data were not obtained. Saline was used occasionally as a control, to determine whether the milk ejection response had become a reflex act conditioned by the routine of making injections.

Two rates of injecting oxytocin were investigated. In the first, an amount of oxytocin in 1 ml. was injected quickly into a jugular vein, which was distended by keeping the vessel occluded until the injection was completed. In the second, and in contrast to the rapid injection in the first method, the needle was inserted into a vein, which had been distended temporarily and then released, and the oxytocin was injected slowly into the streaming blood. The time involved in making the quick injection averaged about 1 to 2 sec.; whereas, the slow injection took place over a period of about 15 to 20 sec. At the completion of each injection, and after the delivery of each 0.1 ml. of solution in the slow-injection series, an attempt was made to draw blood into the syringe. When blood was drawn back freely, this fact was taken as evidence that the needle remained in the vein throughout the injection. When blood did not return freely into the syringe, the data for that particular trial were deleted from consideration.

The effect of injected oxytocin upon subsequent measurements of responses to injected oxytocin was investigated by employing a second injection, either immediately after removal of milk resulting from the primary injection or at one of the next two milking periods.



Three cows in six comparisons were used to evaluate the effect of relaxin[3] in bringing about milk ejection. A single injection of relaxin consisted of 30 or 60 mg., freshly dissolved in saline and administered intravenously.

RESULTS AND DISCUSSION

The relationship between the quantities of oxytocin used at successive milkings and the quantities of complementary milk recovered is shown (Table 1). Generally, similar amounts of oxytocin had been used at the preceding milkings. It is seen that as the amount of oxytocin injected decreased, the quantity, fat percentage, and per cent of complementary milk decreased. The smallest amount of injected oxytocin eliciting a definite milk ejection response was 0.06 I.U. Because there is always a small quantity of milk available in the mammary gland immediately after a control saline injection *(6, 7)*, it was impossible to differentiate between the results from 0.03 I.U and from saline. The complementary milk remaining in the udder after removal of that ejected by injection of 0.06 I.U. was removed by a second injection of 0.25 I.U. oxytocin. The amount of this secondary complementary milk was greater and the fat content lower than one might have expected it to be, according to data presented and reviewed by Koshi (*6*).

The primary complementary milk, expressed as a fraction of the combined complementary milks, averaged 2.53% when the primary injections were saline and 0.03 I.U. oxytocin, but increased to 33.7, 64.0, and 78.6%, respectively, when 0.06, 0.12, and 0.25 I.U. of oxytocin were used as primary injections. In the two later instances, the secondary complementary milk was similar to that expected from normal cows *(7)*. Under these circumstances, 0.12 I. U. of oxytocin may be considered within the physiological range and, as such, may be similar to the amounts released from the posterior pituitary glands prior to milk ejections in normal cows.

Even though this one unique cow was used, the data, nevertheless, may be of great interest and significance. These observations are, insofar as is known to the

[3] Relaxin (The Armour Laboratories preparation No. MN-4; 25-30 guinea pig units per mg.).

TABLE 1

Milk ejection response to various amounts of administered oxytocin

No. of observations	Treatment at experimental milking	Complementary milk		
	(I.U. oxytocin)	*(Av. lb.)*	*(Av % fat)*	*(Av. % of total milk)*
3	10.00	10.0	6.7	89
12	2.50	11.0	5.2	84
8	0.50	8.5	5.1	77
47	0.25	7.2	5.1	76
24	0.18	5.7	4.8	61
36	0.12	4.5	3.5	61
3	0.06	3.1	2.3	46
2	0.03	0.3	2.8	7.9
3	0.00	0.3	3.1	4.2

author, the only ones of their kind. The reliability of the conclusions seems assured by the fact that well over 100 injections were made and the results were highly consistent. There was no suggestion from this animal's reaction that it was in any other way abnormal. The exogenous oxytocin necessary to eject milk from the mammary gland was in all probability similar in amount to that necessary to eject milk from normal milking animals. The presence of this unique cow in the natural population would suggest that attempts should be made to bring this condition about by artificial means, in order to explain such an abnormality and to study milk ejections further. Perhaps many other cows are afflicted with the same disability, but to different degrees. It is important to recognize this condition and to learn something of its variability, in order to prevent or cure it.

The fact that the ejection response was related to the concentration of the hormone administered makes it appear that the myoepithelial tissue, as a whole, does not act according to the all-or-none law, although units of the system may react to various threshold stimuli according to the all-or-none law, as do units of skeletal muscle systems.

The effectiveness of the last previous oxytocin injection(s) and the time interval between injections were important to an accurate assessment of the ejection response to another oxytocin injection. As the time interval between injections was extended from one to two between-milking intervals, quantities of cistern and complementary milk increased, but complementary milk decreased percentage-wise (Table 2). The positive relationship between amount and per cent of complementary milk (Table 1) thus was reversed. As shown (Tables 2 and 3), the quantity of cistern milk generally increased, and the fat per cent of this milk decreased, as the quantity of oxytocin which was last used decreased. In the instance (Table 2), in which no previous oxytocin had been used, 7.0 lb. of cistern and 25.7 lb. of complementary milk were recovered, contrasted to 1.0 lb. of cistern and 10.0 lb. complementary milk at the next milking, from a similar quantity of oxytocin (10 I.U.). Per cent complementary milk increased from 79 to 91 from the first to the second milking.

TABLE 2

Effects on cistern and complementary milk of previous injections of oxytocin

I. U. oxytocin injected at experimental and pre-experimental milkings			Measurements taken at experimental milking				
Two milkings before	One milking before	Experimental milking	Cistern milk[a]		Complementary milk		
			(lb.)	*(% fat)*	*(lb.)*	*(% fat)*	*(% of total)*
0.0	0.0	10.0	7.0	2.3	25.7	12.2	79
10.0	10.0	10.0	1.2	6.7	10.0	6.7	89
0.5	0.0	0.5	6.2	1.5	13.0	4.0	68
0.5	0.5	0.5	2.6	2.8	8.5	5.1	77
2.5	0.0	2.5	4.8	1.6	16.3	5.2	77
2.5	2.5	2.5	2.1	3.2	11.0	5.2	84

[a] Cistern milk—the milk removed before administering oxytocin.

TABLE 3

Influence of amount of oxytocin used at previous milking upon average yield and fat content of cistern milk

Treatment at previous milking	Cistern milk				No. of observations
	Milk yield		Fat content		
(I.U. oxytocin)	*(av. lb.)*	*(range)*	*(av. %)*	*(range)*	
0	5.73	(12.5–2.2)	2.22	(6.3–1.1)	24
0.12–0.67	2.79	(9.5–0.7)	3.29	(5.8–1.9)	148
1–10	1.88	(6.0–0.7)	3.89	(7.6–1.6)	32

The effect of rate of injection of oxytocin upon the ejection response was determined by combining data from trials in which 0.12 and 0.25 I.U. per injection were used. The 0.25 I.U. amounts were injected both as primary and secondary injections, secondary injections being defined as injections made immediately after removing complementary milk resulting from primary injections. Rapidly-injected oxytocin (Table 4) was effective in removing approximately 8% more milk and 13% more butterfat, on the average, than oxytocin injected slowly. Analysis of variance of these data indicated that the quantities of milk and milk fat recovered after fast injections of oxytocin were significantly greater ($P < 0.05$) than the quantities recovered after slow injections. The increased effectiveness of milk ejection brought about by increasing the rate of injecting oxytocin may have been dependent upon the amounts of oxytocin used. After combining the data (Table 2), in which one 0.5 I.U. amount of oxytocin was injected rapidly (0.25 I. U. at previous milking), it is seen that 10.8 lb. (73%) complementary milk was recovered. In a series of ten trials, in which two 0.25 I.U. amounts were injected rapidly within a period of from 5 to 8 min. (0.165 I.U. at previous milking), the complementary milk averaged 10.7 lb. (79%). The series in which two 0.25 I.U. amounts were used was completed 2 mo. after completing the other series and when production had declined from about 18 lb. per day to 15. Although direct comparisons between the two series are somewhat hazardous, because of these differences, it appeared that two 0.25 I.U. amounts (a divided or slow injection) were fully as effective as 0.5 I.U. injected

TABLE 4

Effect of rate of injection of oxytocin upon ejection of complementary milk

Injection[a] rate	Oxytocin treatment used (I.U/injection)		No. of observations	Complementary milk	
	Primary injection	Secondary injection		Milk (lb.)	Fat (lb.)
				(av.)	
Slow	0.25		8	7.6	0.33
Fast	0.25		14	8.4	0.35
Slow	0.12		6	5.6	0.20
Fast	0.12		8	6.2	0.22
Slow		0.25	7	2.2	0.24
Fast		0.25	15	2.2	0.29

[a] Slow injection—1 ml. oxytocin solution injected in approximately 15-20 sec.
Fast injection—1 ml. oxytocin solution injected in approximately 1-2 sec.

TABLE 5

Effect upon milk ejection of reversing the sequence of two oxytocin injections given at one milking period

Oxytocin treatments given at experimental milking		Total complementary milk from both injections of oxytocin	
First injection I.U. oxytocin	Second injection I.U. oxytocin	Milk *(lb.)*	Fat *(lb.)*
		——(*av.*)——	
0.12	0.25	9.0	0.57
0.25	0.12	7.0	0.38
0.25 D[a]	0.25	10.0	0.60
0.25	0.25 D[a]	9.9	0.52

[a] D symbolizes injections that were made slowly, contrasted to injections made rapidly.

as a single or fast injection. Obviously, further work is needed to clarify the relationship between dose levels and rates of injections as they affect milk ejection.

The effectiveness of milk ejection brought about by oxytocin injected as two portions varied according to whether the most effective portion, as judged from independent comparisons, was made first or last. Appropriate amounts of oxytocin were divided into two quantities before injecting them (Table 5). The second injection was given directly after collecting the complementary milk resulting from the first injection. The two injections of a pair were designed to be unequally effective in ejecting complementary milk, either as a result of containing different amounts of oxytocin or of being injected at different rates. The least effective and the most effective injections were used in both possible injection sequences. The results of two series (Table 5) were combined. Approximately 10% more milk and 25% more butterfat were recovered after a quantity of oxytocin was injected as two unequally effective portions, when the most effective portion was used second rather than first in the sequence. These data suggest that the manner in which a cow releases stored oxytocin may affect the efficiency with which milk is ejected. Whittlestone has presented supporting data *(11)*.

Except in one cow (No. 2 in Table 6), very negligible amounts of complementary milk were recovered after giving an intravenous injection of relaxin. It is doubtful that the relaxin complementary milk recovered from Cow Two, when relaxin was the primary treatment, was the result of the action of relaxin. This particular cow, in subsequent observations, was observed to have a second letdown of milk after completion of machine-milking, whenever hand-stripping was carried out. It is reasonable to suspect that the milk attributed to relaxin in the main actually was ejected because of the action of endogenous oxytocin. Results from relaxin (Table 6) were similar to results when saline was used (Table 1). Two of the three animals were injected with saline, which resulted in the recovery of 4.2% complementary milk compared to 5.2% after relaxin injections. It is clear from these data that the effect of relaxin in causing milk ejectioin was very negligible, if present at all.

TABLE 6

Comparison of relaxin and oxytocin in causing milk ejection

Cow No.	Hormone treatment: Sequence of hormones used and amounts of each	Complementary milk							
		Oxytocin induced				Relaxin induced			
		Milk		Fat		Milk		Fat	
		(lb.)	*(% total)*	*(lb.)*	*(% total)*	*(lb.)*	*(% total)*	*(lb.)*	*(% total)*
1	60 Mg. relaxin followed by 1 I.U. oxytocin at same milking	10.5	45.0	1.00	84.0	0.5	2.1	0.02	1.3
1	Same as above	15.0	67.0	0.75	87.0	0.5	2.2	0.01	1.4
2	30 Mg. relaxin followed by 10 I.U. oxytocin at same milking	1.4	11.0	0.24	24.0	1.3	10.0	0.18	20.0
2	10 I.U. oxytocin followed by 30 mg. relaxin at same milking	2.0	17.0	0.26	37.0	0.4	3.5	0.05	7.0
2	30 Mg. relaxin at one milking, 10 I.U. oxytocin at next milking	2.3	19.5	0.46	45.5	1.3	13.5	0.27	36.5
3	10 I.U. oxytocin at one milking, 30 mg. relaxin at next milking	2.0	20.4	0.45	47.3	0.3	5.0	0.02	8.3
	Average	5.5	30.0	0.53	54.0	0.7	6.0	0.09	12.0

ACKNOWLEDGMENTS

The oxytocin and relaxin were furnished through the courtesy of Dr. Irby Bunding of the Armour Laboratories, Chicago.

Dr. James L. Carmon of the Animal Husbandry Department, University of Georgia, performed the analysis of variance of certain of the data utilized in this study.

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IN VITRO STUDIES ON METHANOGENIC RUMEN BACTERIA. II. FERMENTATION OF BUTYRIC AND VALERIC ACID

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SUMMARY

Stabilized enrichment cultures which produced methane from butyric and valeric acids were readily obtained from bovine rumen fluid. All attempts to obtain methanogenic enrichment cultures which convert propionic acid to methane were unsuccessful. Butyric acid was completely oxidized to methane and carbon dioxide. The oxidation of valeric acid was not carried to completion, and in such cultures the propionic acid from the oxidation of valeric acid, coupled with the reduction of carbon dioxide, accumulated in the fermentation medium as an end product. The acetic acid from the oxidation of butyric and valeric acid, coupled with the reduction of carbon dioxide, was readily dissimilated by the cultures. The rate of acetate utilization was equal to or greater than the rate of butyrate and valerate oxidation. The presence of methane bacteria in rumen fluid, which biochemically are closely related to *Methanobacterium suboxydans,* suggests that butyric and valeric acids are the reductants, with carbon dioxide the oxidant as an additional pathway of in vivo formation of rumen methane.

Methane formation resulting from the dissimilation of formic acid and acetic acid by enrichment cultures of rumen bacteria previously has been reported *(7)*. Although reactions involving formic acid and/or its cleavage products may account for considerable quantities of the methane, data have been reported which imply that other substrates, in addition to formate, participate in rumen methanogenesis *(2)*. Thus, on the basis of known reactions of the defined methane bacteria found in marine muds *(9)*, it can be postulated that the reduction of carbon dioxide, coupled with the oxidation of the rumen volatile fatty acids having more than two carbon atoms, contributes to the formation of methane. Accordingly, experiments were designed to determine the capacity of enrichment cultures of methanogenic rumen bacteria to utilize propionic, butyric, and valeric acids as substrates. The data from such experiments are reported herein.

METHODS AND MATERIALS

Bacteriological. The stabilized enrichment cultures of methane bacteria from bovine rumen fluid were established and maintained in the manner previously reported in detail by Oppermann *et al. (7)*. Depending upon the degree of culture activity, from 0.5 to 2.0 m*M* of substrate was added daily to the stabilized cultures incubated at 40° C.

Chemical. The fermentation gases were collected by the displacement of acidified (pH 1.0) saturated aqueous Na_2SO_4, and the volume recorded after adjustment to atmospheric pressure. Absorption-combustion procedures employing a Model No. 35-802 Burrell apparatus were used to determine the composition of

Received for publication December 16, 1957.

the fermentation gases. The volatile fatty acids in the fermentation mixtures were separated and measured quantitatively by the chromatographic technique described in a previous report *(7)*.

RESULTS

Enrichment cultures which fermented butyric or valeric acids to methane were readily obtained from bovine rumen fluid. The endogenous substrates contributed by the rumen fluid inoculum were exhausted after approximately 4 wk. of incubation at 40° C.; thereafter, the enrichment cultures were entirely dependent upon the daily addition of the test substrates. All attempts to establish cultures which fermented propionate were unsuccessful.

The data (Table 1) illustrate the changes in the fatty acid composition of the fermentation medium during the dissimilation of butyric acid by a stabilized

TABLE 1

Fermentation of butyric acid

Incubation time	Acid concentration [a]		Δ Butyric	Δ Acetic	Acetate [b] formed	Rate of utilization [c]	
	Butyric	Acetic				Butyric	Acetic
(hr.)	*(meq/100 ml.)*					*(meq/100 ml/hr)*	
0	0.905	0.825	—	—	—	—	—
4	0.525	0.925	−0.380	+0.100	0.760	0.095	0.165
7	0.325	1.025	−0.200	+0.100	0.400	0.066	0.100
10	0.275	1.375	−0.050	+0.350	0.100	0.017	−0.083
16	0.100	1.185	−0.175	−0.190	0.350	0.029	0.090
24	0.075	1.050	−0.025	−0.135	0.050	0.003	0.023
56	0.040	0.290	−0.035	−0.760	0.070	0.001	0.026

Gas yield = 250 ml. Actual, CO_2: CH_4 = 3 : 5.
Theoretical gas yield = 253 ml. Theoretical, CO_2: CH_4 = 3 : 5.

[a] By analysis.
[b] By calculation $2CH_3(CH_2)_2COOH + 2H_2O + CO_2 \longrightarrow 4CH_3COOH + CH_4$.
[c] By calculation 2Δ butyric ± Δ acetic/time.

rumen enrichment culture. The amount of butyric acid in the medium decreased with time throughout the incubation period. However, the major quantity of the butyrate was dissimilated during the first 7 hr. of incubation. The concentration of acetic acid increased slightly during the period of rapid butyrate fermentation, and thereafter decreased with time throughout the remaining incubation period.

The volume and composition of the gas collected from the fermentation indicate that the butyric acid utilized was completely dissimilated to methane and carbon dioxide. Presumably, the first stage in such a degradation involves the oxidation of butyrate, coupled with the reduction of carbon dioxide, to yield acetate and methane according to Equation 1, as follows:

$$(1) \quad 2CH_3CH_2CH_2COOH + 2H_2O + CO_2 \longrightarrow 4CH_3COOH + CH_4 \quad (9)$$

The final step in the complete degradation of butyrate to methane by enrichment cultures presumably involves the dissimilation of the acetate formed as a reaction

product in the first equation. The dissimilation of acetate to methane presumably proceeds according to Equation 2, as follows:

$$(2) \quad 4CH_3COOH \longrightarrow 4CH_4 + 4CO_2 \quad (8)$$

Thus, the over-all dissimilation of butyric acid by enrichment cultures is depicted as follows:

$$(1) \quad 2CH_3(CH_2)_2COOH + 2H_2O + CO_2 \longrightarrow 4CH_3COOH + CH_4$$
$$(2) \quad 4CH_3COOH \longrightarrow 4CO_2 + 4CH_4$$

Net:

$$(3) \quad 2CH_3(CH_2)_2COOH + 2H_2O \longrightarrow 3CO_2 + 5CH_4$$

As shown (Table 1), the experimental data relating to gas volume and composition are in close agreement with the theoretical values predicted by Equation 3. Thus, the data show that the considerable quantities of acetate formed during the rapid oxidation of butyrate (Equation 1) were degraded to methane and carbon dioxide at a rate only slightly less than that of acetate formation. The data (Table 1) further show that the calculated rate of total acetate utilization generally exceeded the rate of butyrate disappearance.

Because the foregoing data suggest that rumen fluid contains organisms which converted both the butyric acid substrate and the acetic acid intermediate to methane, an experiment was designed to test the hypothesis that butyric and acetic acids are utilized simultaneously by the methane bacteria in a rumen enrichment culture. Accordingly, new cultures were stabilized to a mixed substrate consisting of both acetic and butyric acids. The data presented (Table 2) illustrate

TABLE 2

Simultaneous fermentation of butyric and acetic acids

Incubation time	Acid concentration [a]		Δ Butyric	Δ Acetic	Acetate [b] formed	Rate of utilization [c]	
	Butyric	Acetic				Butyric	Acetic
(hr.)	*(meq/100 ml.)*					*(meq/100 ml/hr)*	
0	1.350	3.390	—	—	—	—	—
3	0.980	3.410	−0.370	+0.020	0.740	0.123	0.240
5	0.440	3.230	−0.540	−0.180	1.080	0.270	0.630
11	0.280	3.180	−0.160	−0.050	0.320	0.027	0.060
21	0.110	2.480	−0.170	−0.700	0.340	0.017	0.104
36	0.020	1.370	−0.090	−1.110	0.180	0.006	0.086

Gas yield = 181 ml. CO_2: CH_4 not determined.
Theoretical gas yield = 210 ml.

[a] By analysis.
[b] By calculation $2CH_3(CH_2)_2COOH + 2H_2O + CO_2 \longrightarrow 4CH_3COOH + CH_4$.
[c] By calculation 2Δ butyric $\pm \Delta$ acetic/time.

the changes in the composition of the fermentation medium of such cultures, and show that the substrates are utilized simultaneously. The rate of total acetate utilization again exceeded the rate of butyrate utilization.

Data relative to the fermentation of valeric acid by rumen enrichment cultures stabilized to this substrate are presented (Table 3). In contrast to the data which show the complete degradation of butyric acid to methane and carbon di-

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TABLE 3

Fermentation of valeric acid

Incubation time	Acid concentration [a]			Change in acids			Reaction products formed [b]		Rate of utilization [c]		
	Valeric	Propionic	Acetic	Δ Valeric	Δ Propionic	Δ Acetic	Propionic	Acetic	Valeric	Propionic	Acetic
(hr.)	*(meq/100 ml.)*								*(meq/100 ml/hr)*		
0	0.910	4.200	0.155	—	—	—	—	—	—	—	—
16	0.490	4.235	0.285	−0.420	+0.035	+0.130	0.420	0.420	0.026	0.024	0.018
40	0.250	4.600	0.180	−0.240	+0.365	−0.105	0.240	0.240	0.010	−0.005	0.014
Σ	—	—	—	−0.660	+0.400	+0.025	0.660	0.660	0.016	0.006	0.016

Gas yield = 85 ml. Actual, CO_2: CH_4 = 2.5 : 1.
Theoretical gas yield = 132 ml. Theoretical, CO_2: CH_4 = 1 : 1.6.

[a] By analysis.
[b] By calculation $2CH_3(CH_2)_3COOH + CO_2 + 2H_2O \longrightarrow 2CH_3CH_2COOH + 2CH_3COOH + CH_4$.
[c] By calculation $\Delta V \pm \Delta P$ or ΔA/time.

TABLE 4

Fermentation of valeric acid by cultures stabilized to butyric acid

Time	Gas Produced	Acid concentration [a]				Reaction products formed [b]		Acid utilization			
		Valeric	Butyric	Propionic	Acetic	Propionic	Acetic	Valeric	Butyric	Propionic	Acetic
(hr.)	*(ml.)*	*(meq/100 ml.)*									
		Trial 1									
0	0	0.67	0.06	0.00	0.83	—	—	—	—	—	—
24	100	0.29	0.00	0.43	0.36	0.38	0.38	0.38	0.06	0.05	0.85
		Trial 2									
0	0	0.96	0.00	0.43	0.36	—	—	—	—	—	—
24	88	0.47	0.00	1.04	0.34	0.49	0.49	0.49	0.00	−0.12	0.51

Gas yield = 188 ml. CO_2: CH_4 = not determined.
Theoretical gas yield = 174 ml.

[a] By analysis.
[b] By calculation $2CH_3(CH_2)_3COOH + CO_2 + 2H_2O \longrightarrow 2CH_3CH_2COOH + 2CH_3COOH + CH_4$.

oxide, the dissimilation of valeric acid did not go to completion in enrichment cultures. The complete degradation of valeric acid by methanogenic bacteria presumably would involve several stages and several species. The oxidation of valerate, coupled with the reduction of carbon dioxide, as mediated by *Methanobacterium suboxydans,* presumably yields methane, propionate, and acetate as follows:

(4) $2CH_3(CH_2)_3COOH+CO_2+2H_2O \rightarrow 2CH_3CH_2COOH+2CH_3COOH+2CH_4$ *(9)*

The data (Table 3) show that valeric acid was readily utilized, as was the acetic acid reaction product resulting from the valerate oxidation. In contrast to these data, the other acid reaction product, propionic acid, was not appreciably utilized.

Additional evidence for the incomplete degradation of valeric acid to methane and carbon dioxide and the accumulation of propionic acid as an end product in rumen enrichment cultures is shown (Table 4). In this instance, valeric acid was used as the substrate for a culture previously stabilized to butyric acid. Propionic acid was not present in the medium immediately after the addition of valeric acid. However, after 24 hr. of incubation, propionic acid was encountered in an amount consistent with that predicted by Equation 4. The second addition of valeric acid resulted in a similar accumulation of propionic acid. In both trials the acetate formed during the oxidation of valerate, and the residual acetate from the previous additions of butyrate, were degraded in quantities equal to or greater than the quantities of valerate utilized. The volume of gas produced in these experiments was consistent with the amounts of valerate and acetate utilized and the concentration of propionate which accumulated.

The effect of formic acid on the degradation of butyric acid by enrichment cultures of rumen methane bacteria is shown (Table 5). In this instance, the culture was stabilized to a substrate consisting of a mixture of formic and butyric acids. The mixed substrate was added at zero time and formic acid was added at 2-hr. intervals during the experiment.

Although butyric, acetic, and formic acids were utilized simultaneously by the culture, the data show that the rate of formate utilization greatly exceeded the rate at which either acetate or butyrate was utilized. A comparison

TABLE 5

Simultaneous fermentation of butyric and formic acid

Time	Substrate added		Volatile acidity			Acetate formed	Acid utilization		
	Butyric	Formic	Butyric	Acetic	Formic		Butyric	Acetic	Formic
(hr.)	*(meq/100 ml.)*								
0	0.54	2.10	0.72	0.62	2.13	—	—	—	—
2	—	1.05	0.67	0.65	2.02	0.10	0.05	0.07	1.16
4	—	0.52	0.62	0.68	1.78	0.10	0.05	0.07	0.76
6	—	0.52	0.55	0.73	1.40	0.14	0.07	0.09	0.90
8	—	1.05	0.46	0.73	1.59	0.18	0.09	0.18	0.86
Σ	0.54	5.24	—	—	—	0.52	0.26	0.41	3.65

Gas yield = 285 ml. CO_2: CH_4 = not determined.
Theoretical yield = 280 ml.

of the rates of butyrate and acetate utilization obtained in this trial and previous trials (Tables 1 and 2) suggests that formic acid was preferentially utilized and that the rates of acetate and butyrate utilization were depressed by the presence of formate.

DISCUSSION

The experiments reported by several investigators have shown that the production of methane from formate by rumen bacteria involves an initial split to CO_2 and H_2 *(1, 3)*, and the subsequent reduction of CO_2 by H_2 *(2)*. Other investigators have emphasized the critical role of H_2 in rumen methanogenesis, and have indicated that the availability of H_2 is a limiting factor in such reactions *(6)*. Data also have been reported which show that the rate of formate production in rumen contents is much less than the potential rate for its conversion, and that other substrates, in addition to formate, participate in the formation of rumen methane *(2)*.

The experimental data presented in the preceding section of this report show that butyric and valeric acids, which are found in the rumen in appreciable quantities, are dissimilated to methane by rumen enrichment cultures. The utilization of these compounds in such cultures is indicative of the presence of methane bacteria in rumen fluid which are biochemically closely related to *Methanobacterium suboxydans (9)*, and suggests that butyric and valeric acids serve as reductants, with CO_2 as the oxidant, in the in vivo formation of rumen methane.

The numerous unsuccessful attempts, in this and previous trials *(7)*, to obtain the utilization of propionate by enrichment cultures, are consistent with the observations of McNeill and Brown *(5)*, and strongly indicate that this component of the volatile acidity is not a substrate in rumen methanogenesis. The accumulation of propionate during the oxidation of valerate by enrichment cultures is additional evidence in support of this viewpoint.

Several categories of evidence show that rumen fluid contains bacteria capable of mediating the formation of methane by the cleavage of acetate *(1, 7)*, the reduction of CO_2 by H_2 *(1, 2)*, and by the reduction of CO_2 coupled with the oxidation of C_4 and C_5 fatty acids (Tables 1 and 4). The exact proportions of methane contributed by these pathways have not been defined. However, the rate of the formate-CO_2-H_2 system, and its apparent depressing effect on acetate utilization *(7)* and possibly on butyrate oxidation (Table 5), suggest that the predominance of the production of methane via the reduction of CO_2 by H_2 is limited only by relative availability of H_2, as previously noted *(6)*. Since CO_2 is not limiting in the rumen, and the quantities of H_2 and formate are not sufficient to account for all the methane formed, it is suggested that acetate, butyrate, and valerate contribute to the formation of rumen methane. In this connection, the data show that the rate of acetate utilization was equal to or greater than that of butyrate or valerate.

Although the pathways of methane formation in the rumen have not been related directly to the chronology of rumen digestion, it is known that the

maximum $CO_2 : CH_4$ ratios in rumen gas *(10)* and maximum volatile acidity of the rumen ingesta *(4)* are encountered within a relatively short period after feeding. Thus, it is possible that the CO_2-H_2 system of methane formation predominates during this early period of rapid fermentation, and that acetate utilization and the reduction of CO_2 coupled with the oxidation of the higher fatty acids, contribute to methane formation late in the digestion cycle when $CO_2 : CH_4$ ratios and volatile acid concentrations are minimum. This postulate has not been tested.

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TECHNICAL NOTES

EFFECT OF ALFALFA ASH AND VALERIC ACIDS ON GROWTH OF DAIRY HEIFERS[1]

Recent work by the Ohio workers *(2,5)* indicates that a combination of rumen factors including valeric acids improved cellulose digestion in the artificial rumen and improved the growth rate of beef cattle in three of four trials. It has been shown that the addition of either alfalfa ash or alfalfa meal to rations containing low-quality roughages will improve the utilization of such rations when fed to beef cattle or sheep *(1,3,4)*. It seemed desirable to evaluate these two factors in growing dairy heifer rations.

Twenty-four yearling dairy heifers (15 Holstein and nine Brown Swiss) were divided into three groups and used in a 150-day feeding trial to study the effect of alfalfa ash and the fatty acids, valeric and isovaleric, upon growth. All heifers were fed ground corn cobs free choice once per day as the sole roughage. In addition, Group 1 heifers were fed a 32% protein soybean oil meal grain mix in sufficient quantities to meet the heifers' need for digestible protein *(6)*; Groups 2 and 3 were fed the same grain mix supplemented with either 25.7 g. of alfalfa ash or 1.0 g. of valeric and 0.27 g. of iso-valeric acids per pound of grain mix, respectively.

The influence of the supplements on the feed consumption, growth rate, and feed efficiency of the heifers is shown (Table 1). Neither the alfalfa ash nor the combination of fatty acids appeared to affect the consumption of corn cobs significantly.

The supplementation of the control ration with either alfalfa ash or the combination of fatty acids increased the growth rate of the heifers significantly. Both supplements appeared to exert their greatest effect during the first 30-day period. The average daily gains of the heifers were 1.71, 2.30, and 2.03 lb. for Groups 1, 2, and 3, respectively, for the first 30-day period. Average daily gains of the heifers were 1.14, 1.26, and 1.32 lb. per day for Groups 1, 2, and 3, respectively, for the last 120 days of the experiment. These latter differences in growth rate were approaching significance (5% level).

The results of this experiment indicate that both alfalfa ash and the combination of fatty acids exerted a beneficial effect upon the growth rate of dairy heifers fed corn cobs as a roughage. Growth and feed consumption data collected in this experiment would indicate that both alfalfa ash and the fatty acids improved the growth rate of the heifers through some mechanism other than increased appetite.

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[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2115.

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TABLE 1

Effect of alfalfa ash and a combination of valeric and iso-valeric acids on the growth rate of dairy heifers

Group	Feed	Daily feed	Initial weight	Final weight	Daily gain	Feed/lb gain
				(lb.)		
1	Corn cobs Ration 1	14.9 3.6	679	867	1.26	14.7
2	Corn cobs Ration 2	14.7 3.6	678	898	1.47	12.4
3	Corn cobs Ration 3	15.0 3.6	679	899	1.47	12.7
L.S.D.—5%		N.S.			0.09	N.S.

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EFFECT OF HEAT ON THE CENTRIFUGAL SEDIMENTATION OF MILK SERUM PROTEINS

Some diversity of opinion exists among investigators regarding the effect of heat on the subsequent removal of the milk serum proteins by high-speed centrifugation. There appears to be agreement, however, that no significant amount of serum proteins in raw skimmilk is removed by a centrifugal force of about 30,000 times gravity for periods of up to 1 hr. Ramsdell and Whittier *(3)* centrifuged heated and unheated skimmilk for various periods of time in an air-driven, bowl-rotor ultracentrifuge *(2)*, analyzed the supernatant liquid, and plotted the grams of acid-coagulable nitrogen per 100 g. of liquid against total nitrogen. They interpreted the parallelism of the lines for heated and unheated milk to mean "that no albumin or globulin was thrown out" under their conditions of centrifuging, and that "these whey proteins were not coagulated." The difference between the two lines was constant at about 0.09% nitrogen.

This represents the heat-denatured serum proteins, which are determined by the difference between the acid-precipitable fractions in heated and unheated milk. Since the heated sample is also centrifuged in these types of experiments, the data do not distinguish between serum proteins removed by acid coagulation and those removed by centrifugation. Thus, the parallelism of the lines for heated and unheated milks, obtained by plotting as described above, can not be used as a criterion of the removal of serum proteins from heated milk by centrifugation.

Early papers *(1, 2, 3)* have not reported the removal of enough nitrogen by centrifugation of heated samples to account for all of the casein, let alone the serum proteins. Recently, Sullivan *et al. (4)* have shown that a considerable quantity of the serum proteins is removed by high-speed centrifugation after heating to 200° F. for 1 min.

The data reported herein are compatible with Sullivan's results, in that they show the serum proteins to be rendered centrifugable by heating to 90° C. for 15 min.

Heated and unheated skimmilks were centrifuged for 1 hr., using an average effective centrifugal force of 123,600 times gravity. The noncentrifuged samples and the supernatant portion of the centrifuged samples were analyzed for total nitrogen and acid-precipitable nitrogen. The results, expressed as grams of nitrogen per 100 g. of liquid, are as follows:

Component	Raw milk	Heated milk
Noncentrifuged samples		
Total nitrogen	0.5830	0.5830
Acid-precipitable nitrogen	0.4480	0.5300
Nonacid-precipitable nitrogen	0.1350	0.0530
Serum protein nitrogen (0.1350 − 0.0530) = 0.0820		
Centrifuged samples		
Total nitrogen	0.1435	0.0794
Acid-precipitable nitrogen	0.0081	0.0264
Nonacid-precipitable nitrogen	0.1354	0.0530
Serum protein nitrogen (0.1354 − 0.0530) = 0.0824		

It is readily seen that the nonacid-precipitable fractions in raw and heated milks were different, but that they were not changed by centrifuging, remaining as 0.135% in the raw milk and 0.053% in the heated milk. The difference is 0.082%, representing the heat-denaturable serum protein fraction. However, the fact that this difference was constant before and after centrifuging does not indicate anything regarding the changes in serum protein brought about by heating. Only 0.0264 g. of acid-precipitable nitrogen per 100 g. of liquid remained in the heated sample after centrifuging. This shows that enough nitrogen was removed to account

$$\text{for } 68\% \left[100 \times \frac{0.082 - 0.0264}{0.082} \right] \text{ of the serum}$$

proteins, plus all of the casein. If some of the casein was noncentrifugable in the heated sample, then more than 68% of the serum protein was removed. Possibly, a longer centrifuging time would have removed an additional amount.

It is of interest to note that about 98%

$$\left[100 \times \frac{0.448 - 0.0081}{0.448} \right] \text{ of the casein in the raw}$$

milk was removed under these centrifugal conditions, whereas the serum proteins were unaffected.

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OUR INDUSTRY TODAY

FUNCTIONS OF THE DEPARTMENT OF AGRICULTURE IN RELATION TO DEFINITIONS, STANDARDS, AND GRADES OF CHEESE[1]

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The Food and Drug Administration is concerned with definitions and standards of identity and wholesomeness of the product; whereas, grade standards relate to refinements of marketable quality.

In addition to developing grade standards, we cooperate with the General Services Administration in the preparation and revision of federal specifications for all dairy products. These specifications are used by all civilian federal agencies in their procurement needs, exclusive of the Price Support Program. The Defense Department uses the federal specifications in many instances, but they also prepare and use their own military specifications on certain products.

Fluid milk and milk products which are covered by the Grade A program are under the jurisdiction of the U. S. Public Health Service and similar agencies.

An important function of the Agricultural Marketing Service, U. S. Department of Agriculture, is to aid in the orderly marketing of agricultural products through the development of grade standards which serve as a basis for measuring and evaluating quality and provide a common trading language between buyer and seller. In formulating definitions of dairy products, in the establishment of grade standards, our language may go beyond the wording of Congressional and Federal Food and Drug Administration definitions, but never in conflict with their provisions. Under Public Law 733, known as the Agricultural Marketing Act of 1946, and previous similar laws, the Secretary of Agriculture has been authorized and directed to promulgate grade standards for agricultural products. Likewise, the Secretary of Agriculture is required under the Administrative Procedure Act of 1946, Public Law 414, to publish all grade standards and any revisions thereof in the Federal Register, to afford opportunity for interested parties to submit their views, comments, and suggestions, signifying their approval or objection.

Inspection and grade certification of farm products based upon established quality standards have become a fixed part of our agricultural economy for many years.

Specifically, the Dairy Division, AMS, is charged with the responsibility of developing grade standards for manufactured dairy products, including cheese. Since the main interest and grading activity for cheese relates to Cheddar cheese, we will confine our remarks to this product.

History of Grade Standards for Cheddar Cheese

The original grade standards for Cheddar cheese were developed in the early twenties after thorough and careful investigation. These grade standards were promulgated in January, 1923, under the Food Products Inspection Law. They were designed on the basis of the score-card system, with certain maximum ratings given to the various quality factors. During World War II we abandoned the score-card method of prorating the score to the various quality factors, and employed a more direct method of classifying each quality factor. The revised grade standards embodying the direct method of grading were promulgated in May, 1943, and were revised slightly in December, 1950. Based upon approximately 15 years of extensive experience in grading Cheddar cheese during the war and after, a comprehensive revision of the grade standards was issued in May, 1956.

Fundamentals of Grade Standards

Grade standards, to be of maximum value, should encompass the full range of marketable quality and should be based on factors that can be uniformly applied. They should reflect the essential commodity characteristics, so that they will be useful to industry and to users of the product. With respect to manufactured dairy products they should, insofar as possible, reflect the quality of the raw milk and/or hygiene of manufacture.

Although there is no overlapping of quality between grades, a certain range or latitude in quality is allowed in each grade. As one goes down the grade scale, the latitude in quality within each grade widens progressively. For example, if one were to divide a pyramid

[1] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

outline into four equal segments—the peak representing Grade AA, the next segment Grade A, then Grade B, and the fourth segment at the base Grade C—the matter of quality tolerance between grades would become quite apparent. Grade standards, to be realistic and effective, do not change according to market conditions or from season to season—the same considerations apply to the interpretation and application of the standards through grading activity.

Grade standards should be designed to lend themselves to a high degree of standardization when applied by experienced personnel under effective supervision.

Present Grade Standards

The present grade standards which were adopted in May, 1956, are more complete, specific, and informative than earlier standards. Four grades are established, namely, U. S. Grade AA, U. S. Grade A, U. S. Grade B, and U. S. Grade C. These grades are based on four quality factors, i.e., flavor, body and texture, color, finish and appearance. Characteristics or defects, including intensity, of each quality factor, according to the degree of curing, are classified. The final U. S. Grade for any given vat of cheese is established on the basis of the lowest rating of any one of the quality characteristics.

Condensed as well as detailed tables are used to tabulate characteristics, according to the degree of curing, thereby providing quick reference for the specific grades. Proper application and use of these grade standards should aid the industry in marketing and merchandising Cheddar cheese.

Grading Cheddar Cheese

The Dairy Inspection and Grading Branch is charged with the responsibility of determining the grade in accordance with the U. S. Standards for Cheddar cheese. In the grading of Cheddar cheese offered for possible sale to the Government under the price support program, many quality problems are encountered.

A summary (Table 1) of the grading data was tabulated from a representative group of grading certificates covering Cheddar cheese offered for sale to the Commodity Credit Corporation (CCC) during the period July, 1956, through June, 1957. During this period, the Government purchased 206,015,303 lb. of U. S. Grade A paraffined cheese. The summary represents a total of 55,410,455 lb. (approximately 1,665 carlots) offered for possible sale to the Commodity Credit Corporation, of which 91.0% met the purchase requirements of U. S. Grade A. The remainder consisted of 8.0% U. S. Grade B, 1.0% U. S. Grade C, and less than 0.01% was Below Grade. The sampling represents one-fourth (24.5%) of the paraffined cheese offered for sale to the CCC.

The certificates were selected at random, by states, in proportion to the total poundage offered. In addition, they were selected in groups from the same company on the same day's or subsequent days' gradings, from which was recorded the poundage of each U. S. grade, including Below Grade, together with the corresponding poundage for each defect. When more than one characteristic was reported by the grader, for any given vat, the first one listed was recorded as the defect most likely responsible for the cheese being graded down.

TABLE 1

Summary of reasons for grade-outs of paraffined Cheddar cheese, July, 1956—June, 1957

Flavor (18.0%)[a]		Body and texture (53.1%)[a]		Color (2.3%)[a]		Finish and appearance (26.6%)[a]	
	(%)		(%)		(%)		(%)
Utensil	22.7	Gassy	47.8	Mottled	65.7	Blistered paraffin	38.3
Acid	20.4	Open	28.9	Seamy	18.1	Wet rind	29.6
Yeasty	19.1	Weak	6.4	Acid-cut	7.4	Mold under paraffin	12.8
Old milk	12.3	Sweet holes	3.7	Salt spots	7.0	Checked rind	4.5
Weedy	6.2	Crumbly	3.7	All others	1.8	High edges	2.8
Fruity	5.6	Slitty	2.6	Unnatural		Cracked rind	2.3
Onion	5.3	Pinny	2.4	Dull		Sour rind	1.9
Whey taint	4.1	All others	4.5			Scaly paraffin	1.9
All others	4.3	Short				Weak rind	1.5
Bitter		Corky				Huffed	1.4
Metallic		Mealy				All others	3.0
Feed		Pasty				Soiled surface	
Sour		Coarse				Rough surface	
Flat		Curdy				Lopsided	
Lipase						Irregular bandage	
						Checked paraffin	
						Broken bandage	
						Rind rot	
						Soft spots	

[a] Percentage of grade-outs in each group.

TABLE 2

Summary of reasons for grade-outs of rindless Cheddar cheese, July, 1956—June, 1957

Flavor (37.5%)[a]		Body and texture (81.2%)[a]		Color (2.8%)[a]		Finish and appearance (6.8%)[a]	
	(%)		(%)		(%)		(%)
Utensil	21.3	Gassy	53.3	Mottled	53.3	Rough surface	27.4
Acid	19.2	Open	25.3	Seamy	30.3	Moisture under wrapper	26.4
Yeasty	12.9	Short	6.6	Acid-cut	8.0	Loose wrapper	20.3
Old milk	11.4	Slitty	3.4	Faded	5.2	Mold under wrapper	17.6
Weedy	10.9	Weak	2.7	All others	3.2	Huffed	2.6
Fruity	8.3	Coarse	2.7	Extraneous matter		Broken wrapper	2.4
Bitter	8.1	Corky	2.0	Salt spots		Checked surface	2.2
All others	7.9	Crumbly	1.5			Soiled surface	0.6
Whey taint		Pinny	1.1			Wrinkled wrapper	0.5
Onion		Mealy	0.7				
Sour		All others	0.7				
Lipase		Sweet holes					
Rancid		Pasty					
		Curdy					

[a] Percentage of grade-outs in each group.

Cheddar cheese from 16 states was included in the paraffined cheese summary, with the majority of the cheese offered being from nine midwestern and southern states.

A separate tabulation (Table 2) of all the rindless Cheddar cheese offered to CCC was prepared for the same period. This summary was prepared on the basis of reporting multiple defects for any given vat of cheese. When more than one defect was shown on the certificate, for any vat, each was recorded and the same poundage tabulated for each defect. Although this would not change the total poundage shown for each grade, the poundage for each defect would more clearly reflect the importance of each characteristic.

Six states were represented in this summary of the rindless Cheddar cheese, comprising a total of 14,972,750 lb. offered of which 12,447,604 lb., or 83.1%, graded U. S. Grade A. Of this amount, 12,141,138 lb. were purchased. Of the total graded, 15.9% was U. S. Grade B, 0.9% U. S. Grade C, and less than 0.1% was Below Grade.

With respect to flavor defects for paraffined Cheddar cheese, utensil, acid, yeasty, and old milk flavors constituted 74.5% of the off flavors; for body and texture defects, gassy and open amounted to 76.7%; for color defects, mottled and seamy amounted to 83.8%; and for finish and appearance defects, blistered paraffin, wet rind, and mold under paraffin amounted to 80.7% (Table 1).

In the summary of the rindless Cheddar cheese, it is interesting to note the degree of uniformity of defects with the paraffined cheese, except in finish and appearance characteristics, which are not on a comparable basis. This is even more striking when it is considered that the rindless cheese was offered in fewer states and produced by fewer companies than the paraffined cheese.

Owing to the duplication of poundage for the multiple defects reported, it was not possible to compute the exact percentages for each quality factor in arriving at the grade-out breakdown. Therefore, the percentages reported below for each quality factor total more than 100%. In the rindless Cheddar cheese group, 37.3% of the grade-outs possessed faulty flavor characteristics; body and texture accounted for 81.2%; color, 2.7%, and finish and appearance, 6.8%.

In the off-flavor category for rindless Cheddar cheese, utensil, acid, yeasty, old milk, weedy, fruity, and bitter flavors were 92.1%; for body and texture defects, gassy and open were 78.6%; for defects in color and appearance, rough surface, moisture under wrapper, loose wrapper, and mold under wrapper were 91.1% (Table 2).

Quality Improvement

It is quite generally known that a substantial amount of cheese offered for sale to the Government is pregraded, or at least screened, before being officially graded by federal or federal-state graders. Also, that up to three-fourths of the Cheddar cheese production is not exposed to such grading and that a significant portion of this cheese would probably not qualify for U. S. Grade A. Moreover, when grading cheese of from 10 to 21 days old, as in the case of the Price Support Program, a certain amount of grading failures is inescapable. Consequently, we can not begin to assume that 91% of all of the paraffined Cheddar cheese produced in this country would qualify for U. S. Grade A. Actually, we have a long way to go toward improving the over-all quality of Cheddar cheese in this country.

One of the prime objectives of grade standards is to facilitate trading and to stimulate quality improvement, uniformity and stability of product. Basically, quality improvement in Cheddar cheese involves these considerations: 1. Improve the quality of milk on the farm. 2. Improve plant sanitation. 3. Use good starters or cultures. 4. Use better manufacturing methods.

Too much emphasis can not be placed upon the importance of the quality of the raw milk. Only a few states have adequate requirements for the production of quality milk for manufacturing purposes; others have few or no applicable regulations. Improvement in the quality of milk for manufacturing purposes will tend to narrow the range of quality within grades; hence, will help bring about a general uplifting of the quality standards for Cheddar cheese.

There are no short cuts to quality improvement. The achievement of a sound quality program will require a lot of hard work and team effort of everyone. We can build our quality program on solid rock or we can attempt to build it on quicksand. But we feel confident that the leaders of the cheese industry will help lay a strong foundation, so that our quality structure and grade standards for Cheddar cheese will yield maximum returns.

LABOR-SAVING METHODS FOR MAKING CHEDDAR CHEESE [1]

H. E. Walter, A. M. Sadler, and C. D. Mitchell

Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

Labor-saving procedures and equipment for making Cheddar cheese are urgently needed to compensate for increased labor costs. Also, means for improving the flavor and uniformity of cheese are needed to increase consumption.

Conventional methods for making Cheddar cheese require much hand labor, particularly during the cheddaring or matting process. Several commercial cheese companies, equipment makers, and state agricultural experiment stations have attempted to develop a stirred-curd method that would require appreciably less hand-labor and consequently reduce manufacturing costs. Some workers achieved a fair degree of success but, in general, the resultant product had a more open texture than that characteristic of Cheddar cheese. Satisfactory results were not obtained when these proposed methods were tested in our laboratory; the texture of the cheese was too open.

Earlier, promising results were obtained by the Department when salted curd was hooped in salted whey. This finding resulted in the development of a short-time method, which was reported in 1953. However, there were recognized objections to certain parts of that method. It required special types of cheesemaking equipment. Also, approximately one-half of the whey was unusable because it contained 4% added salt.

Australian investigators carried out extensive studies with our method and concluded that it was not commercially acceptable, particularly because of the hooping in salted whey. However, using some of the basic principles of our method, they developed a method that was accepted by several of their commercial factories, and cheese was made for export.

Continued research in our laboratory resulted in several important changes in the original procedure. The amount of salted whey for hooping the curd was reduced significantly, and most of the special equipment was eliminated. "A Simplified Short-Time Method for Making Cheddar Cheese from Pasteurized Milk" was reported in 1955 (ARS-73-11). The method will be briefly described:

The starter employed is a mixture of a conventional lactic starter and the heat- and salt-tolerant *Streptococcus durans,* which are propagated separately. The starter and rennet are added to pasteurized milk at 88° F. The curd is cut and cooked to 100° F. in a conventional cheese vat. The whey is drained off and the curd is salted. The salted curd is hooped at about 110° F. in a small volume of either salted whey or salted water, drained under pressure for a few minutes, and pressed overnight in a conventional cheese press. The method requires only 3 hr. until the curd is pressed, instead of the conventional 5½ to 6½ hr.

The quality of the cheese made by this method is excellent. It has a clean, mild flavor and an extremely close texture. However, some persons have objected to this close texture and the lack of mechanical openings. Also, some have objected to a need for additional hooping equipment.

In view of these objections, it seemed desirable to investigate possible changes that might be made in the simplified method. It was decided to approach the problem on the basis that: *(1)* A saving of labor is more important than a saving of time; *(2)* some mechanical openings in Cheddar cheese are not undesirable, and *(3)* a labor-saving method

[1] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

should deviate as little as possible from conventional methods.

A rather simple change has been made in the conventional method which seems to offer a practical means for saving much hand-labor. The new method has been tested repeatedly on a pilot-plant scale. It has not been tested on a commercial scale; therefore, its final evaluation must await results obtained in commercial cheese factories.

With the new method, the conventional 7-hr. method of making Cheddar cheese from pasteurized milk, as described in USDA Circular No. 880, is followed, except during the period from draining the whey to milling the curd. At the usual time for draining off the whey, the mixture of curd and whey is pumped with a positive-action rotary pump into a cheesecloth-lined, perforated curd-retention and matting device which has been placed in a tank. (See description which follows.) The mixture of curd and whey is discharged at the top of the device. The curd falls to the bottom, forming a layer under the whey, and most of the free air entrapped between the curd particles is released and comes to the surface of the whey. When pumping has been completed, the cheesecloth is folded over the curd, a perforated top plate is placed on the covered curd, weights equal to 30 lb. per square foot are added, and the whey is drained from the tank. The whey continues to drain from the curd and the curd mats for 2 hr. without being turned. Then the weights, top plate, and cheesecloth are removed and the large block of curd is cut into ¾-in. slabs for milling. Conventional procedures are followed in milling, salting, hooping, dressing, and pressing the curd. Most of the tedious hand-labor required during the 2¼-hr. matting period is eliminated. Further, the cheese vat is available for reuse when pumping is completed.

Cheese made with this method has had the same general characteristics and composition as cheese made with the conventional method. It has had a typical Cheddar cheese flavor, few mechanical openings, and a firm, pliable body. During the early months of curing, more flavor developed and the body became smoother and more waxy. Time has not permitted a determination of the amount of flavor development upon extending aging.

The stainless steel curd-retention and matting device used in developing this method was 22 in. wide, 49 in. long, and 24 in. deep. It was perforated with ¼-in. circular holes every 2½ in. on centers throughout the lower 12 in. The upper half was not perforated. The top and bottom plates were perforated. The device was large enough to hold the curd from 4,000 lb. of milk. The tank was slightly larger than the matting device and it was deep enough to insure complete coverage of the curd with whey throughout the pumping period. It was so constructed that the excess whey could be drained off near the top.

Numerous variations from the conventional procedure have been tried, but so far this modification has yielded the best results. Research is continuing, to determine more precisely the effects of variations in individual steps of the new procedure.

THE FUNCTIONS OF THE FOOD AND DRUG ADMINISTRATION IN RELATION TO DEFINITIONS AND STANDARDS FOR CHEESE [1]

L. M. Beacham

Food and Drug Administration, Washington, D. C.

The authority for promulgating standards of identity, quality, and fill of container under Section 401 of the Food, Drug, and Cosmetic Act, and the nature of such standards, are discussed. Guiding principles as they have been set forth in interpretative regulations are then explained, dealing with such matters as required and optional ingredients and proper use of the names of standardized foods upon their labels.

Next is given a brief description of the procedure followed in establishing food standards under the Hale Amendment to the Act, and then the procedure followed when the proceedings require a public hearing.

Up to the present, sixty standards applicable to cheese, cheese foods, cheese spreads, and other standardized cheese products have been established. These can be found in Code of Federal Regulations, Title 21, Chapter 1, Part 19.

Important revisions in the cheese standards since 1955 are cited. The most notable of these is permission to use 0.2% sorbic acid on certain cheeses in the form of slices or cuts in consumer-size packages with the label statement "Sorbic acid added to retard mold growth" or "Sorbic acid added as a preservative." The cheeses in which this is permitted are: Cheddar, Washed Curd, Colby, Granular, Swiss, Provolone, Asiago fresh, Semisoft Part-Skim, Pas-

[1] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

teurized Process Pimiento Cheese, Pasteurized Process Cheese Spread, Gruyère, Brick, Muenster, Monterey, High-moisture Jack, Caciocavallo Siciliano, Semisoft Cheeses, Pasteurized Process Cheese, and Pasteurized Process Cheese Food.

Sodium or calcium propionate, or both, also may be used in concentrations up to 0.3% in pasteurized process cheese, pasteurized process pimiento cheese, pasteurized process cheese food, and pasteurized process cheese spread. A label statement similar to either of those just described must be used.

A discussion follows of recent proposals for new cheese standards, e.g., Ricotta, Part-Skim Ricotta, Samsoe, Mozzarella, Part-Skim Mozzarella, Grated American Cheese Food, partially Creamed Cottage Cheese, and Nuworld Cheese. The latter is the only one of these for which a standard of identity has become effective. It is a blue-mold type of cheese, but is made with a white-mold mutant developed by X-ray exposure of blue mold. The petition for a standard for partially Creamed Cottage Cheese has been denied after public hearings. The proposal was for a Cottage Cheese with other added dairy ingredients to give a product having between 0.5 and 2.0% fat. At a hearing, the testimony showed that many persons could not clearly distinguish the product from plain Cottage Cheese or from Creamed Cottage Cheese with 4% fat. A standard for such a product did not appear likely to promote honesty or fair dealing in the interest of consumers.

The remaining proposals for standards for cheese and cheese products are still under consideration and may require public hearings. The standards for Ricotta and Part-Skim Ricotta have been stayed, because of objections from certain importers that the name Ricotta properly belongs to an imported whey cheese. Those for Mozzarella and Part-Skim Mozzarella have not become effective because of objections by interested parties that the moisture limit is too low. The proposed standard for Samsoe has been stayed because of objections of packers of domestic Swiss Cheese, who feel that because Samsoe resembles Swiss cheese so closely it should have the same moisture and fat limits, i.e., 41 and 43%, respectively. For grated American Cheese Food, the published standard has been stayed because of objections from Kraft Foods, Western Condensing Co., and Foremost Dairies to the failure of the standard to list dried whey as a permitted ingredient, and objections of Kraft Foods to other features of the standard, as well.

OUR ASSOCIATION

PROGRAM

FIFTY-THIRD ANNUAL MEETING

of the

AMERICAN DAIRY SCIENCE ASSOCIATION

NORTH CAROLINA STATE COLLEGE

RALEIGH

June 16–19, 1958

Program Committee

N. P. RALSTON, Michigan, *Chairman*
C. F. HUFFMAN, Michigan, *Ex Officio*
F. J. BABEL, Indiana
S. P. MARSHALL, Florida
L. R. FRYMAN, Illinois
J. W. POU, North Carolina

GENERAL PROGRAM

Saturday, June 14

Executive Board Committee Meetings, *Alumni Building*

Sunday, June 15

9:00 A.M. **Meeting of the Executive Board,** *Alumni Building Conference Room*

Monday, June 16

9:00 A.M. **Meeting of the Executive Board,** *Alumni Building Conference Room*

9:00 A.M. **Registration,** *William Neal Reynolds Coliseum*

5:30 P.M. **Past Presidents' Dinner,** *Student Union*

7:30 P.M. **Informal Reception,** *William Neal Reynolds Coliseum*

Prelude

National Anthem

Invocation

REVEREND O. B. WOOLDRIDGE, *Coordinator of Religious Affairs, N. C. State College*

Welcome

Dr. C. H. Bostian, *Chancellor, North Carolina State College*

Introduction of American Dairy Science Association Officers and Directors and Distinguished Guests

Informal Reception

Tuesday, June 17

6:30 P.M. Barbecue, *Freshman Football Field*

8:30 P.M. Entertainment. W. D. Carmichael, Jr., *Vice-President, University of North Carolina, Master of Ceremonies. William Neal Reynolds Coliseum*

Wednesday, June 18

10:15 A.M.–12:30 P.M.

General Business Session of Association, D. V. Josephson, *Chairman. William Neal Reynolds Coliseum*

4:00–5:30 P.M.

Membership Committee Meeting. *Room 110, Polk Hall*

8:00 P.M.

Recognition Program, *William Neal Reynolds Coliseum*

MANUFACTURING SECTION

Tuesday, June 17

8:30–11:15 A.M.

Section A. Dairy Chemistry
Kilgore Hall, Room 159

Section B. Dairy Bacteriology
Scott Hall, Room 212

11:30 A.M.–12:00

Manufacturing Section Business Meeting
Kilgore Hall, Room 159

1:30– 3:30 P.M.

Section A. Joint with Production and Extension Sections
Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Recommendations
Williams Hall Auditorium

SECTION B. Milk Enzymes
Kilgore Hall, Room 159

3:45– 4:45 P.M.

MANUFACTURING SECTION BUSINESS MEETING
Kilgore Hall, Room 159

5:30– 6:30 P.M.

STUDENT AFFILIATES AND FACULTY ADVISORS' MEETING
Williams Hall Auditorium

Wednesday, June 18

8:00–10:00 A.M.

SECTIONS A AND B
Symposium: Latest Developments in the Heat and Vacuum Treatment of Milk
Williams Hall Auditorium

10:15 A.M.–12:30 P.M.

GENERAL BUSINESS SESSION OF THE ASSOCIATION
D. V. JOSEPHSON, *Chairman*
William Neal Reynolds Coliseum

2:00– 5:00 P.M.

SECTION A. Dried, Concentrated, and Modified Milks
Scott Hall, Room 212

SECTION B. Dairy Industry
Kilgore Hall, Room 159

JOINT WITH PRODUCTION AND EXTENSION SECTIONS
Panel on Education. The Humanistic and Social Studies in a Technical or Scientific Curriculum
William Neal Reynolds Coliseum

Thursday, June 19

8:15–11:45 A.M.

SECTION A. Milk Proteins
Kilgore Hall, Room 159

8:30–11:30 A.M.

SECTION B. Cheese
Scott Hall, Room 212

PRODUCTION SECTION

Tuesday, June 17

8:00 A.M.–12:00

SECTION A. **General Nutrition**
Riddick Auditorium, Room 242

SECTION B. **Milk Secretion and Physiology**
Withers Auditorium, Room 118–119

10:00 A.M.–12:00

JOINT WITH EXTENSION SECTION
Special Topic: What Dairy Records Should Contain to Be of Most Value to Dairymen
(SEE EXTENSION SECTION)
Broughton Auditorium, Room 111

1:30– 3:30 P.M.

SECTION A. **Rumen Physiology**
Riddick Auditorium, Room 242

SECTION B. **Artificial Insemination**
Withers Auditorium, Room 118–119

JOINT WITH MANUFACTURING AND EXTENSION SECTIONS
Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Recommendations
(SEE MANUFACTURING SECTION)
Williams Hall Auditorium

3:40–4:30 P.M.

Production Section Business Meeting
Riddick Auditorium, Room 242

4:30–5:30 P.M.

JOINT WITH EXTENSION SECTION

JOINT COMMITTEE REPORTS
(SEE EXTENSION SECTION)
Riddick Auditorium, Room 242

Wednesday, June 18

8:00–10:00 A.M.

SECTION A. **Silage and Pasture**
Riddick Auditorium, Room 242

SECTION B. **Artificial Insemination**
Withers Hall Auditorium, Room 118–119

10:15 A.M.–12:30 P.M.

GENERAL BUSINESS SESSION OF THE ASSOCIATION
D. V. JOSEPHSON, *Chairman*
William Neal Reynolds Coliseum

2:00–5:00 P.M.

JOINT WITH EXTENSION SECTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Quality and Its Evaluation
Williams Hall Auditorium

SECTION B. **Reproduction and Genetics**
Riddick Auditorium, Room 242

JOINT WITH MANUFACTURING AND EXTENSION SECTIONS
Panel on Education. The Humanistic and Social Studies in a Technical or Scientific Curriculum
William Neal Reynolds Coliseum

5:00–6:30 P.M.

STUDENT AFFILIATES AND FACULTY ADVISORS' MEETING
Williams Hall Auditorium

5:00–6:00 P.M.

JOINT WITH EXTENSION SECTION

MEETING OF SUPERINTENDENTS OF OFFICIAL TESTING
(SEE EXTENSION SECTION)
Broughton Auditorium, Room 111

Thursday, June 19

8:00 A.M.–12:00

SECTION A. **Calf Nutrition and Physiology**
Riddick Auditorium, Room 242

SECTION B. **Genetics**
Withers Auditorium, Room 118–119

8:30–11:30 A.M.

JOINT WITH EXTENSION SECTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Utilization
(SEE EXTENSION SECTION)
Williams Hall Auditorium

EXTENSION SECTION

Tuesday, June 17

9:00–10:00 A.M.

Dairy Records
Broughton Auditorium, Room 111

10:00 A.M.–12:00

JOINT WITH PRODUCTION SECTION
Panel—What Dairy Records Should Contain to Be of Most Value to Dairymen
GENE MEYER, *Moderator and Discussion Leader*
Broughton Auditorium, Room 111

1:30–3:30 P.M.

Teaching Methods
Stimulating Positive Thinking
E. A. FAILS, *North Carolina State College*
Presentation and Discussion of Exhibits
Broughton Auditorium, Room 111

JOINT WITH MANUFACTURING AND PRODUCTION SECTIONS
Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Regulations
(SEE MANUFACTURING SECTION)
Williams Hall Auditorium

3:30–4:30 P.M.

EXTENSION SECTION BUSINESS MEETING
Broughton Auditorium, Room 111

4:30–5:30 P.M.

JOINT PRODUCTION AND EXTENSION BUSINESS MEETING
Joint Committee Reports
Riddick Auditorium, Room 242

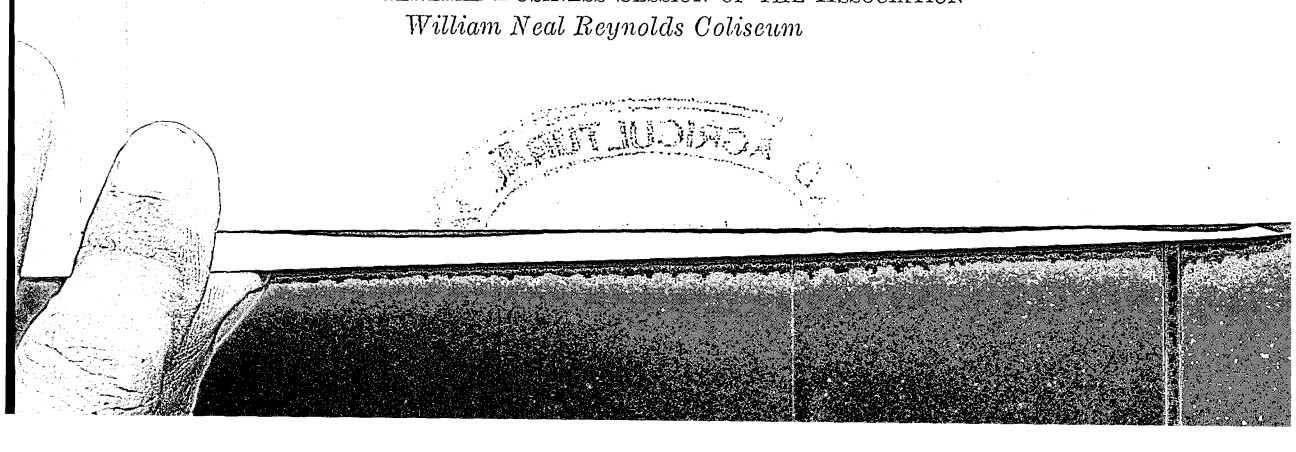
Wednesday, June 18

8:00–10:00 A.M.

Dairy Records and Herd Management
Broughton Auditorium, Room 111

10:15–12:30 P.M.

GENERAL BUSINESS SESSION OF THE ASSOCIATION
William Neal Reynolds Coliseum



2:00–4:00 P.M.

4-H Program and Older Youth Program
Broughton Auditorium, Room 111

2:00–5:00 P.M.

JOINT WITH PRODUCTION AND MANUFACTURING SECTIONS
Panel on Education. The Humanistic and Social Studies in a Technical or Scientific Curriculum
William Neal Reynolds Coliseum

JOINT WITH EXTENSION SECTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Quality and Its Evaluation
Williams Hall Auditorium

5:00 P.M.

JOINT WITH PRODUCTION
Meeting of Superintendents of Official Testing
Broughton Auditorium, Room 111

Thursday, June 19

8:30–11:30 A.M.

JOINT WITH EXTENSION SECTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Utilization
Williams Hall Auditorium, Room 111

ENTERTAINMENT

GENERAL PROGRAM

Monday, June 16 — 7:30 P.M.—Welcome and Informal Reception
William Neal Reynolds Coliseum

Tuesday, June 17 — 6:30 P.M.—Barbecue
Freshman Football Field

Wednesday, June 18 — 8:00 P.M.—Recognition Program
William Neal Reynolds Coliseum

8:30 P.M.—Entertainment
William Neal Reynolds Coliseum

WOMEN'S PROGRAM

Tuesday, June 17 — 1:30 P.M. to 4.45 P.M.—Visit to North Carolina Museum of Art and Tea at Governor's Mansion

Wednesday, June 18 — 9:00 A.M. to 4:30 P.M.—Tour to Chesterfield Cigarette Factory, Duke University, Morehead Planetarium, and University at Chapel Hill

AGRICULTURAL

Thursday, June 19 — 9:00 A.M.—**Open House,** *School of Textiles, N. C. State College*

10:30 A.M.—**Farewell Coffee Hour,** *Chancellor's Home*

CHILDREN'S PROGRAM

(See Separate Program)

Children 3-5 Years*

Tuesday, June 17 — 9:00 A.M.—**Supervised Care.** *Fairmount Methodist Church* to 11:30 A.M.

1:30 P.M.—**Supervised Care.** *Fairmount Methodist Church* to 5:00 P.M.

Wednesday, June 18 — 9:00 A.M.—**Supervised Care.** *Fairmount Methodist Church* to 4:30 P.M.

Thursday, June 19 — 9:00 A.M.—**Supervised Care.** *Fairmount Methodist Church* to 12:00

Children 6 Years and Older**

Monday, June 16 — 7:00 P.M.—**Get-Acquainted Program** to 9:00 P.M. *Frank Thompson Gymnasium*

Tuesday, June 17 — 9:00 A.M.—**Team and Large Group Games** to 11:30 A.M. *Frank Thompson Gymnasium*

1:30 P.M.—**Team and Large Group Games** to 5:00 P.M. *Frank Thompson Gymnasium*

Wednesday, June 18 — 9:00 A.M.—**All Day Outing and Picnic** to 4:30 P.M. *Pullen Park*

7:30 P.M.—**Rotative Party** to 9:30 P.M. *Frank Thompson Gymnasium*

Thursday, June 19 — 9:00 A.M.—**Swimming**—*Frank Thompson Gymnasium* to 12:00

—**Movies**—*College Union*

* Under supervision of experienced kindergarten teachers.
** Under supervision of Department of Industrial and Rural Recreation.

MANUFACTURING SECTION

Tuesday, June 17

8:30–11:15 A.M.

Section A. **Dairy Chemistry**
B. H. Webb, *Chairman*
Kilgore Hall, Room 159

M1. The density of milk at low temperatures. P. D. WATSON, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M2. The interrelation between conductivity, per cent lactose, and freezing point of milk. I. I. PETERS AND R. R. SHRODE, *Texas Agricultural and Mechanical College.*

M3. Influence of vacuum pasteurization upon the freezing points, total solids, and concentration of milk. J. T. LAZAR, JR. AND R. W. HENNINGSON, *Clemson Agricultural College.*

M4. Sedimentation of whole milk heated in the Mallory small tube heat exchanger. H. K. WILSON AND E. O. HERREID, *University of Illinois.*

M5. The effect of treatment of raw milk on heat-liberated hydrogen sulfide. T. KRISTOFFERSEN, *Ohio State University.*

M6. A study of factors which cause variations in protein-reducing value of fluid milk. J. T. CARDWELL AND F. H. HERZER, *Mississippi State College.*

M7. Isolation of milk phospholipids and determination of their polyunsaturated fatty acids. L. M. SMITH AND E. L. JACK, *University of California.*

M8. Determination of ammonia and the lower volatile amine concentrations in milk by solvent extraction and gas-liquid chromatography. C. L. HANKINSON AND D. D. COLE, *Ohio Agricultural Experiment Station and The Ohio State University.*

M9. A method for the determination of citric and lactic acids in dairy products. D. R. FRAZEUR, *Purdue University.*

M10. The distribution between the fat phase and the aqueous phase of certain antioxidants added to whole milk. J. MARTINEZ-MATEO, W. L. CHOATE, AND L. F. EDMONDSON, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M11. Properties of interesterified butteroil. H. D. WEIHE AND G. R. GREENBANK, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M12. Characterization of the volatile and non-volatile fatty acids obtained by a Reichert-Meissl-Polenske distillation. ARUN SENGUPTA, W. F. SHIPE, AND A. C. DAHLBERG, *Cornell University.*

M13. Vitamin B_{12} in whey and casein fractions of milk separated by coagulation with acid and rennet. E. B. COLLINS AND MAKOTO YAGUCHI, *University of California.*

8:30–11:15 A.M.

SECTION B. **Dairy Bacteriology**
F. J. BABEL, *Chairman*
Scott Hall, Room 212

M14. Observations on bulk quality on truck vs. individual farm tank samples. H. V. ATHERTON, *University of Vermont.*

M15. The quality of manufacturing-grade milk handled in farm bulk tanks. W. S. LAGRANGE AND F. E. NELSON, *Iowa State College.*

M16. Observations on bacteria counts of farm bulk-cooled milk. H. V. ATHERTON, *University of Vermont.*

M17. Preserving the activity of lactic cultures. BURDET HEINEMANN, *Producers Creamery Co., Springfield, Mo.*

M18. Production, distribution, and use of frozen, active lactic cultures. J. C. SIMMONS AND D. M. GRAHAM, *Clemson Agricultural College.*

M19. The effect of temperature on acid production by *Streptococcus lactis* and *Streptococcus cremoris.* J. WOLK AND R. P. TITTSLER, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M20. Acceleration of cottage cheese making by the use of starter culture stimulants. M. L. SPECK AND R. A. LEDFORD, *North Carolina State College.*

M21. The influence of stimulatory peptides on proteolysis of milk by lactic streptococci. M. L. SPECK AND W. T. WILLIAMSON, *North Carolina State College.*

M22. Inhibitory effect of nisin upon various organisms. K. M. SHAHANI, *University of Nebraska.*

M23. A test for detecting lactic bacteriophage using 2,3,5–triphenyltetrazolium chloride. B. J. LISKA, *University of Florida, and* H. E. CALBERT, *University of Wisconsin.*

M24. The association between *Bacterium linens* and an oxidative type yeast in the ripening process of surface-ripened cheeses. R. L. MAGINNIS AND J. F. CONE, *Pennsylvania State University.*

M25. The effect of penicillin upon the morphology and gram-staining characteristics of *Streptococcus lactis* and *Streptococcus thermophilus.* R .W. BAUGHMAN AND F. E. NELSON, *Iowa State College.*

M25A. Taxonomy of carbon dioxide-producing lactic acid streptococci found in mixed strain starter cultures. W. E. SANDINE, P. R. ELLIKER, AND A. W. ANDERSON, *Oregon Agricultural Experiment Station.*

11:30 A.M.–12:00

MANUFACTURING SECTION BUSINESS MEETING
F. J. BABEL, *Chairman*
Kilgore Hall, Room 159

1:30–3:30 P.M.

SECTION A. JOINT WITH PRODUCTION AND EXTENSION SECTIONS

Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Recommendations

STUART PATTON, *Chairman*
L. A. BLACK, *Moderator*
Williams Hall Auditorium

Use of insecticides in the production of milk. A. W. LINDQUIST, *Entomology Research Division, USDA, Beltsville, Maryland*

Pesticide residues in dairy practice. F. A. VORHEES, JR., *Food and Drug Administration, Washington, D. C.*

Prevention of antibiotics in milk: Present status. D. C. GROVE, *Division of Antibiotics, Food and Drug Administration, Washington, D. C.*

The use of markers in veterinary preparations for the detection of antibiotics in milk. R. E. HARGROVE, *Eastern Utilization Research and Development Division, USDA;* R. D. PLOWMAN, *Animal Husbandry Research Division, USDA, and* W. WRIGHT, *Food and Drug Administration, Washington, D. C.*

1:30–3:30 P.M.

SECTION B. **Milk Enzymes**

F. J. BABEL, *Chairman*
Kilgore Hall, Room 159

M26. Some observations on lipase activity. B. L. HERRINGTON AND E. S. GUTHRIE, *Cornell University.*

M27. Effect of light, agitation, acids, and rennet upon lipolytic activity in milk. B. J. DEMOTT, *University of Tennessee.*

M28. The acid degree values and 1-monoglyceride content of normal and rancid milk. R. G. JENSEN AND M. E. MORGAN, *Storrs (Conn.) Agricultural Experiment Station.*

M29. Distribution and partial purification of milk lipases. Z. SAITO, W. J. HARPER, AND I. A. GOULD, *Ohio State University.*

M30. Rates of lipolysis in milk as affected by duration of activation by shaking. N. P. TARASSUK AND M. YAGUCHI, *University of California.*

M31. The possible role of xanthine oxidase in spontaneously oxidized milk. A. E. WOODS, L. W. AURAND, AND W. M. ROBERTS, *North Carolina State College.*

M32. Study of the phosphatase test for milk pasteurization. J. B. MICKLE, P. J. MAGNINO, JR., AND R. D. MORRISON, *Oklahoma State University.*

M33. Stability of disodium phenyl phosphate in carbonate-bicarbonate buffer. R. W. HENNINGSON, *Clemson Agricultural College*

3:45–4:45 P.M.

MANUFACTURING SECTION BUSINESS MEETING
F. J. BABEL, *Chairman*
Kilgore Hall, Room 159

5:00–6:30 P.M.

STUDENT AFFILIATES AND FACULTY ADVISORS' MEETING
W. L. SLATTER, *Chairman*
Williams Hall Auditorium

Wednesday, June 18

8:00–10:00 A.M.

SECTIONS A AND B
Symposium: Latest Developments in the Heat and Vacuum Treatment of Milk
B. H. WEBB, *Chairman*
Williams Hall Auditorium

Physical-chemical aspects. R. JENNESS, *University of Minnesota,* and S. PATTON, *Pennsylvania State University.*

Engineering aspects. C. W. HALL, *Michigan State University.*

Bacteriological aspects. F. W. BARBER, *National Dairy Products Corporation, Long Island, N. Y.*

Problems involved in flavor removal. W. M. ROBERTS, *North Carolina State College.*

10:15 A.M.–12:30 P.M.

GENERAL BUSINESS SECTION OF THE ASSOCIATION
D. V. JOSEPHSON, *Chairman*
William Neal Reynolds Coliseum

2:00–5:00 P.M.

SECTION A. **Dried, Concentrated, and Modified Milks**
F. J. BABEL, *Chairman*
Scott Hall, Room 212

M34. A method to improve the "sinkability" of dried whole milk. D. H. BULLOCK AND W. C. WINDER, *University of Wisconsin.*

M35. The effect of raw milk quality on the flavor stability of dried milk. U. S. Ashworth and C. C. Prouty, *State College of Washington.*

M36. Identification of some compounds affecting the flavor of nonfat dry milk. Richard Bassette and Mark Keeney, *University of Maryland.*

M37. Further investigations on the stale-flavor components in dried whole milk. W. W. Nawar, H. E. T. Dall, A. S. Ganguly, S. H. Lombard, and R. McL. Whitney, *University of Illinois.*

M38. The migration of phosphatides in the processing of dairy products. I. Foam and spray-dried whole milk. G. R. Greenbank, *Eastern Utilization and Research Development Division, USDA, Washington, D. C.*

M39. Effect of superheating on the baking properties of nonfat dry milk. L. V. Rogers, H. A. Anderson, and C. F. Hufnagel, *Eastern Utilization and Research Development Division, USDA, Washington, D. C.*

M40. Free fat in foam-dried whole milk. A. Tamsma, L. F. Edmondson, and H. E. Vettel, *Eastern Utilization and Research Development Division, USDA, Washington, D. C.*

M41. High milk-protein bread. L. V. Rogers and Helen H. Welton, *Eastern Utilization and Research Development Division, USDA, Washington, D. C.*

M42. A study of the process of gelation, flocculation, and caramelization as related to heat stability in concentrated milk. R. N. Giroux, *Ecole de Laiterie Provinciale, St. Hyacinthe, Que.*, and H. E. Calbert and A. M. Swanson, *University of Wisconsin.*

M43. Ultra-high temperature sterilization and aseptic canning of concentrated milk using pilot equipment. G. H. Hartman, *White House Milk Co., Inc., Manitowoc, Wisconsin,* and D. V. Alstrand, *American Can Co., Maywood, Illinois.*

M44. Factors pertaining to the stability of ascorbic acid and vitamin B_6 in modified milk products. O. F. Garrett, R. W. Sharp, and Jean Kleckner, *M & R Dietetic Laboratories, Inc., Columbus, Ohio.*

M45. Relation of type of fat in modified sterilized milk products to stability of vitamin B_6 and to physiological response when fed to experimental rats. L. S. Baur and O. F. Garrett, *M & R Dietetic Laboratories, Inc., Columbus, Ohio.*

2:00–5:00 P.M.

Section B. **Dairy Industry**
H. F. Judkins, *Chairman*
Kilgore Hall, Room 159

M46. Market agency education assists dairy operators in solving engineering and cost problems. A. L. RIPPEN, *Michigan State University.*

M47. The influence of rapid cooling on the development of a rancid flavor in milk produced by bulk system. P. E. JOHNSON AND R. L. VON GUNTEN, *Oklahoma State University.*

M48. A radiometric method for measuring stainless steel corrosion. W. J. HARPER, *Ohio State University.*

M49. Factors of valve design associated with the homogenization of milk. J. J. BETSCHER, W. J. HARPER, I. A. GOULD, AND D. A. SEIBERLING, *Ohio State University.*

M50. Influence of bias in different age groups on consumer preferences of chocolate milk. H. S. WILLARD AND W. R. THOMAS, *University of Wyoming.*

M51. The production of a high-nitrogen, low-lactose product from whey using an automatic pH control system. D. R. ARNOTT, *Purdue University,* and S. PATTON AND E. M. KESLER, *Pennsylvania State University.*

M52. The manufacture of improved quality sherbets. W. S. ARBUCKLE AND E. A. DAY, *University of Maryland.*

M53. A centrifugal ultrafiltration method for studying the soluble constituents of ice cream. B. W. THARP AND P. G. KEENEY, *Pennsylvania State University.*

M54. The influence of selected ingredients on the melt-down of vanilla ice cream. P. J. ROBERTS AND D. R. FRAZEUR, *Purdue University.*

M55. The interrelationships of heat treatment, solids-not-fat, and calcium chloride to the yield of cottage cheese. C. W. DILL, W. M. ROBERTS, AND R. J. MONROE, *North Carolina State College.*

M56. Effect of skimmilk heat treatments on cottage cheese manufacture. D. B. EMMONS, A. M. SWANSON, AND W. V. PRICE, *University of Wisconsin.*

M57. Determination of the curd-making properties of cottage cheese type nonfat dry milk. W. K. STONE AND P. M. LARGE, *Virginia Agricultural Experiment Station, Blacksburg.*

M58. Effects of starters on acidity and firmness of cottage cheese coagulum. D. B. EMMONS AND W. V. PRICE, *University of Wisconsin.*

2:00–5:00 P.M.

JOINT WITH PRODUCTION AND EXTENSION SECTIONS

Panel on Education. The Humanistic and Social Studies in a Technical or Scientific Curriculum

J. W. SHIRLEY, G. A. GULLETTE, V. A. RICE, *North Carolina State College;* F. J. DOAN, *Pennsylvania State University,* and J. H. ERB, *The Borden Company, Columbus, Ohio.*
William Neal Reynolds Coliseum

Thursday, June 19

8:15–11:45 A.M.

SECTION A. **Milk Proteins**
STUART PATTON, *Chairman*
Kilgore Hall, Room 159

Invited Papers:

Biological significance of the milk proteins. B. L. LARSON, *University of Illinois.*

The separation and characterization of the components of α-casein. T. L. MCMEEKIN, *Eastern Utilization Research and Development Division, USDA, Philadelphia.*

M59. Aggregation of calcium caseinate by heat and its reversal. C. A. ZITTLE, *Eastern Utilization Research and Development Division, USDA, Philadelphia.*

M60. Separation of a calcium-soluble fraction of casein from isoelectric casein. K. K. FOX, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M61. Additional evidence of a stable complex between β-lactoglobulin and α-casein. J. C. TRAUTMAN AND A. M. SWANSON, *University of Wisconsin.*

M62. Electrophoretic and ultracentrifugal analysis of casein complex size fractions. F. A. HECKMAN, W. L. CHOATE, AND T. F. FORD, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M63. The voluminosity of casein complex in milk, and reconstituted sediments. C. H. WHITNAH AND W. D. RUTZ, *Kansas State College.*

M64. Size memory of casein colloid particles. W. L. CHOATE, F. A. HECKMAN, AND T. F. FORD, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M65. Effects of accelerated electron irradiation on the casein of skimmed milk. J. R. BRUNNER AND R. C. NICHOLAS, *Michigan State University.*

M66. Effects of proteins on agglutination of fat globules. ALAN KENYON AND ROBERT JENNESS, *University of Minnesota.*

M67. Sedimentation of proteins in milk: A comparative study of the influence of heat, concentration, and rennin action. I. S. VERMA AND C. W. GEHRKE, *University of Missouri.*

M68. Phospholipid and casein interactions. S. M. HUSAINI AND A. M. SWANSON, *University of Wisconsin.*

M69. Optical ultracentrifugal resolution of casein complex particles in milk. T. F. FORD, LORRAINE W. KLIPP, G. A. RAMSDELL, AND W. L. CHOATE, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

8:30–11:30 A.M.

SECTION B. **Cheese**
F. J. BABEL, *Chairman*
Scott Hall, Room 212

M70. Application of tritium and carbon^{14}-labeled compounds in cheese ripening. A. WOLIN AND F. V. KOSIKOWSKI, *Cornell University.*

M71. Characteristic Cheddar cheese flavor in relation to hydrogen sulfide and free fatty acids. T. KRISTOFFERSEN AND I. A. GOULD, *Ohio State University.*

M72. Identification of volatile carbonyls from Cheddar cheese. E. A. DAY AND MARK KEENEY, *University of Maryland.*

M73. Methyl ethyl ketone and other carbonyl compounds in Cheddar cheese. R. SCARPELLINO AND F. V. KOSIKOWSKI, *Cornell University.*

M74. Further studies on Cheddar cheese-making by the Australian short method. P. A. DOWNS, *University of Nebraska.*

M75. The effect of salt on acid development in Cheddar cheese. H. E. WALTER, A. M. SADLER, C. D. MITCHELL, AND R. E. HARGROVE, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M76. Acidity and age of natural cheese as factors affecting the body of pasteurized process cheese spread. N. F. OLSON, D. G. VAKALERIS, W. V. PRICE, AND S. G. KNIGHT, *University of Wisconsin.*

M77. Interrelationships between pH, populations of *Propionibacterium shermanii,* levels of free fatty acids, and the flavor ratings of Swiss cheese. F. E. KURTZ, J. A. HUPFER, E. A. CORBIN, R. E. HARGROVE, AND H. E. WALTER, *Eastern Utilization Research and Development Division, USDA, Washington, D. C.*

M78. Continuous mechanical process for draining and hooping Blue cheese curd. D. M. GRAHAM, *Clemson Agricultural College.*

M79. Selection of *Penicillium* strains for Blue cheese. D. M. GRAHAM, *Clemson Agricultural College.*

M80. Development of a domestic Feta cheese. C. EFTHYMIOU AND J. F. MATTICK, *University of Maryland.*

M81. The vitamin B_6 potency of cheese. I. L. HATHAWAY, *University of Nebraska.*

PRODUCTION SECTION

Tuesday, June 17

8:00 A.M.–12:00

SECTION A. **General Nutrition**

S. P. MARSHALL, *Chairman*

Riddick Auditorium, Room 242

P1. Formulation and feeding of purified diets to dairy cattle. J. H. BYERS AND J. R. STAUBUS, *University of Illinois.*

P2. The effect of all-hay vs. all-silage rations on dry matter intake of lactating dairy cows; moisture and pH as factors affecting appetite. D. HILLMAN, C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, *Michigan State University.*

P3. The effect of various additives on dry matter intake of heifers fed either alfalfa hay or silage. J. P. EVERETT, JR., C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, *Michigan State University.*

P4. Studies on forage-concentrate ratios for milk production. J. L. MORRIS, E. GAINER, AND R. F. DAVIS, *University of Maryland,* and C. R. RICHARDS, *University of Delaware.*

P5. Effect of level of feed intake using hay-grain ratios on feed utilization of dairy cows. C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, *Michigan State University.*

P6. Effect of supplemental fat in high-energy rations on hot-weather performance of producing dairy cows. R. R. HARRIS, R. E. LEIGHTON, AND I. W. RUPPEL, *Texas A & M College.*

P7. The effect of feeding tetra alkylammonium stearate to milking cows. A. C. LINNERUD, J. D. DONKER, AND J. B. WILLIAMS, *University of Minnesota.*

P8. The effect of certain measured environmental influences on the butterfat yield of Holstein-Friesian cattle. E. E. STARKEY, E. L. CORLEY, AND E. E. HEIZER, *University of Wisconsin.*

P9. Effect of feeding thyropotein to dairy heifers. V. SINGH AND J. D. DONKER, *University of Minnesota.*

P10. Methods of adjusting roughage intake data of dairy cows for differences in body size. R. E. MATHER AND A. A. RIMM, *New Jersey Agricultural Experiment Station.*

P11. A comparison of feeding corn cobs and a liquid urea-molasses-mineral mix with and without ethyl alcohol to yearling dairy heifers. J. P. BATES, JR., D. R. JACOBSON, J. W. RUST, AND D. M. SEATH, *University of Kentucky.*

P12. A proposed method for the measurement of the respiratory exchange and energy expenditure of grazing animals. W. P. FLATT, D. R. WALDO, AND J. F. SYKES, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P13. Digestibility of orchard grass hays as influenced by two methods of nitrogen application. R. A. MARKLEY, J. L. CASON, AND B. R. BAUMGARDT, *New Jersey Agricultural Experiment Station.*

P14. The effect of high ash materials upon the use of the fecal chromogens method of determining digestibility. C. R. RICHARDS AND W. G. CROTHERS, *University of Delaware.*

P15. Trace mineral supplementation for dairy cows. E. G. MOODY, J. M. CONOVER, AND A. P. COOK, *Arizona State College.*

P16. Magnesium sulfate, grain, or alfalfa hay as sources of magnesium to calves fed whole milk. J. W. THOMAS AND M. OKAMOTO, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P17. The determination of methoxyl in the presence of carbohydrates. W. C. JACOBSON AND H. G. WISEMAN, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P18. Determination of sugars in silage with micro-Somogyi copper reagent. H. G. WISEMAN, J. C. MALLACK, AND W. C. JACOBSON, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P19. The effect of adding water to the concentrate mix on maximum milking rate, average milking rate, and eating rate. E. W. HUPP AND R. C. LEWIS, *Michigan State University.*

8:00 A.M.–12:00

SECTION B. **Milk Secretion and Physiology**
N. L. JACOBSON, *Chairman*
Withers Hall Auditorium, Room 118–119

P20. The effect of some weather factors on daily milk and fat production. F. N. DICKINSON, *USDA,* and H. W. NORTON AND R. W. TOUCHBERRY, *University of Illinois.*

P21. A study of milk flavors. C. F. FOREMAN, E. W. BIRD, F. E. NELSON, W. S. ROSENBERGER, AND M. R. LAMBERT, *Iowa State College.*

P22. The effect of thyroprotein on metabolic and productive function of lactating Jersey cattle exposed to hot and cool weather. J. E. JOHNSTON, G. A. HINDERY, G. T. SCHRADER, AND F. B. HAMBLIN, *Louisiana Agricultural Experiment Station.*

P23. The effects of high levels of vitamin D_2 fed prepartum on the incidence of parturient paresis in dairy cattle. J. C. DELL AND B. R. POULTON, *University of Maine.*

P24. A calcium lactate–aluminum hydroxide preparation as a milk fever preventive. G. M. WARD, *Colorado State University.*

P25. Production of polyvalent antibodies in milk—absorption and prophylactic and therapeutic value in calves. MOHAMMAD SARWAR, W. E. PETERSEN, AND BERRY CAMPBELL, *University of Minnesota.*

P26. Antitryptic factor and antibody absorption. W. E. PETERSEN, MOHAMMAD SARWAR, AND BERRY CAMPBELL, *University of Minnesota.*

P27. Milk residues associated with use of experimental animal systemic insecticides. J. E. CASIDA, R. P. NIEDERMEIER, J. B. KNAAK, W. C. DAUTERMAN, H. R. KRUEGER, AND F. W. PLAPP, *University of Wisconsin.*

P28. A comparison of the orange G dye and Kjeldahl methods for determining milk protein. J. M. TREECE, L. O. GILMORE, AND N. S. FECHHEIMER, *Ohio Agricultural Experiment Station and The Ohio State University.*

P29. Milk preservative for solids-not-fat determinations. M. L. NELSON, M. N. DEUTSCH, AND C. M. CLIFTON, *University of Minnesota.*

P30. In vitro cultivation of bovine mammary gland in tissue cultures. K. E. EBNER AND B. L. LARSON, *University of Illinois.*

P31. Progress report on experimental stimulation of milk secretion. C. W. TURNER AND H. L. RUPPERT, JR., *University of Missouri.*

P32. Optimal hormone levels and lactation. C. E. GROSVENOR AND C. W. TURNER, *University of Missouri.*

P33. Protein-bound iodine and I^{131} extrapolation technique as methods of determining thyroid activity in dairy cows. F. B. HAMBLIN, J. E. JOHNSTON, AND G. SCHRADER, *Louisiana Agricultural Experiment Station.*

P34. Turnover of I^{131}-labelled thyroxine in cattle. B. N. PREMACHANDRA, G. W. PIPES, AND C. W. TURNER, *University of Missouri.*

P35. Efficiency of absorption of thyroxine in thyroprotein from the gastrointestinal tract of lactating dairy cattle. J. P. MIXNER AND H. D. LENNON, JR., *New Jersey Agricultural Experiment Station.*

P36. Magnitude and variations in plasma PBI values in Ayrshire cattle at the West Virginia Experiment Station. R. O. ASPLUND, G. A. MCLAREN, AND I. D. PORTERFIELD, *West Virginia University.*

P37. An isotope dilution technique for the determination of adrenocorticotropic hormone secretion rate in dairy cattle. W. F. WILLIAMS, *University of Maryland.*

P38. Some physiological responses of the bovine to dihydrotachysterol and parathyroid extract administration. K. A. KENDALL AND K. E. HARSHBARGER, *University of Illinois.*

10:00 A.M. 12:00

JOINT SESSION WITH EXTENSION SECTION

Selected Topic: What Dairy Records Should Contain to Be of Most Value to Dairymen

(SEE EXTENSION SECTION)

Broughton Auditorium, Room 111

1:30–3:30 P.M.

SECTION A. **Rumen Physiology**

S. P. MARSHALL, *Chairman*

Riddick Auditorium, Room 242

P39. The release of ammonia nitrogen from uric acid, urea, and certain amino acids in the presence of rumen microorganisms. C. G. LOOPER AND O. T. STALLCUP, *Arkansas Agricultural Experiment Station.*

P40. The B-vitamin and N requirements of *Bacteroides succinogenes,* an important ruminal cellulolytic bacterium. HILDA CHU AND M. P. BRYANT, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P41. Metabolism and absorption of butyrate-l-C^{14} by the perfused rumen. R. E. MCCARTHY, J. C. SHAW, JEANNE MCCARTHY, AND S. LAKSHMANAN, *University of Maryland.*

P42. The effect of KNO_2 and KNO_3 on in vitro cellulose digestion by bovine rumen microorganisms. V. H. VIEBROCK AND C. P. MERILAN, *University of Missouri.*

P43. Volatile fatty acids production and absorption in the perfused rumen. C. L. DAVIS, R. E. BROWN, J. R. STAUBUS, AND W. O. NELSON, *University of Illinois.*

P44. The effects of various feeds on volatile fatty acid production in vitro. R. G. HINDERS AND G. M. WARD, *Colorado State University.*

P45. Effect of ethanol on rumen fermentation. T. R. LEWIS, R. S. EMERY, AND J. P. EVERETT, *Michigan State University.*

P46. Efficiency of rumen fermentation as affected by corn and adaptation to ration. R. S. EMERY, C. K. SMITH, T. R. LEWIS, AND JUNE DEHATE, *Michigan State University.*

P47. Physical and chemical changes in bovine saliva and rumen liquid with different hay-grain ratios. C. K. SMITH, R. S. EMERY, R. M. GRIMES, C. F. HUFFMAN, AND C. W. DUNCAN, *Michigan State University.*

P48. Relationships between acetate, propionate, and butyrate metabolism in ruminant liver slices. G. I. PRITCHARD AND S. B. TOVE, *North Carolina State College.*

1:30–3:30 P.M.

SECTION B. **Artificial Breeding**
N. L. JACOBSON, *Chairman*
Withers Hall Auditorium, Room 118–119

P49. Fertility of bull semen stored for one and two days at 5° C. in 20% yolk-citrate-glycine-glucose extenders. R. H. FOOTE AND D. C. YOUNG, *Cornell University,* and H. O. DUNN, *New York Artificial Breeders' Cooperative, Inc., Ithaca.*

P50. Prolonged survival of bovine sperm in the Illini variable temperature diluent. N. L. VANDEMARK AND F. D. BARTLETT, JR., *University of Illinois.*

P51. A comparison of two diluents in single-service glass ampules for routine artificial insemination of cattle. A. F. MCFEE, L. J. BOYD, AND E. W. SWANSON, *University of Tennessee.*

P52. Fertility of bull semen stored at room temperature for six days. H. O. DUNN, *New York Artificial Breeders' Cooperative,* and R. H. FOOTE, *Cornell University.*

P53. Livability of bovine spermatozoa at room temperatures in a coconut milk-citrate-calcium carbonate diluent. CHARLES NORMAN, C. E. JOHNSON, AND I. D. PORTERFIELD, *West Virginia University.*

P54. Comparison of skimmilk-egg yolk-glycerol, egg yolk-citrate-glycerol, and homogenized whole milk-glycerol as extenders for bovine semen. B. W. PICKETT, M. C. JOHNSTONE, W. A. COWAN, AND PAUL HELLER, *University of Connecticut.*

P55. Sperm reserves of dairy bulls as determined by depletion trials and post-slaughter sperm counts. J. O. ALMQUIST, R. P. AMANN, AND W. T. O'DELL, *The Pennsylvania State University.*

P56. Sperm production and fertility of dairy bulls at high collection frequencies with varying degrees of sexual preparation. J. O. ALMQUIST, E. B. HALE, AND R. P. AMANN, *The Pennsylvania State University.*

P57. The effects of daily ejaculation on sperm output, fertility, and libido of dairy bulls. H. D. HAFS, R. S. HOYT, AND R. W. BRATTON, *Cornell University.*

1:30–3:30 P.M.

JOINT SESSION WITH MANUFACTURING AND EXTENSION SECTIONS
Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Recommendations
(SEE MANUFACTURING SECTION)
Williams Hall Auditorium

3:40–4:30 P.M.

SECTION BUSINESS MEETING
S. P. MARSHALL, *Chairman*
Riddick Hall Auditorium, Room 242

4:30–5:30 P.M.

JOINT SESSION WITH EXTENSION SECTION
Joint Committee Reports
(SEE EXTENSION SECTION)
Riddick Auditorium, Room 242

Wednesday, June 18

8:00–10:00 A.M.

SECTION A. **Silage and Pasture**
S. P. MARSHALL, *Chairman*
Riddick Auditorium, Room 242

P58. Microbiological and chemical changes in grass silage. C. W. LANGSTON, HERBERT IRVIN, C. H. GORDON, CECELIA BOUMA, H. G. WISEMAN, C. G. MELIN, AND L. A. MOORE, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P59. Preservatives for alfalfa silage. R. J. BAKER AND H. H. VOELKER, *South Dakota Agricultural Experiment Station.*

P60. Effects of wilting and preservatives on composition of legume-grass silage in miniature silos. R. S. ALLEN AND C. F. FOREMAN, *Iowa State College.*

P61. Toxic gases from silage. L. C. WANG, R. H. BURRIS, AND R. P. NIEDERMEIER, *University of Wisconsin.*

P62. The nutrient losses and feeding values of wilted and direct-cut forages stored in bunker and tower silos. C. H. GORDON, C. G. MELIN, W. G. JACOBSON, H. G. WISEMAN, E. A. KANE, AND J. C. DERBYSHIRE, *Dairy Cattle Research Branch,* and J. R. MCCALMONT AND D. T. BLACK, *Agricultural Engineering Research Branch, USDA, Beltsville, Maryland.*

P63. Feeding value for milking cows of oat silages cut at three stages of maturity. F. A. MARTZ, C. H. NOLLER, D. L. HILL, AND M. W. CARTER, *Purdue University*

P64. Soilage or silage for lactating dairy cows? C. F. FOREMAN, R. S. ALLEN, AND A. R. PORTER, *Iowa State College.*

P65. Milk production correlated with quality and quantity of daily herbage intake. A. L. BRUNDAGE AND W. J. SWEETMAN, *Alaska Agricultural Experiment Station.*

P66. Forage and milk yields from alfalfa under three different harvesting systems. R. M. PORTER AND S. R. SKAGGS, *New Mexico Agricultural Experiment Station.*

8:00–10:00 A.M.

SECTION B. **Artificial Insemination**

N. L. JACOBSON, *Chairman*
Withers Hall Auditorium, Room 118–119

P67. Fertility of frozen semen stored in plastic and glass ampules and of first and second ejaculates. H. H. BRUGMAN AND R. P. SCHMIDT, *University of Maine.*

P68. The addition of progesterone and estrogen to bovine semen. R. E. STAPLES, W. HANSEL, R. H. FOOTE, AND H. O. DUNN, *Cornell University.*

P69. The destiny of "sampler" bulls. R. B. BECKER AND P. T. DIX ARNOLD, *Florida Agricultural Experiment Station.*

P70. Results from breeding cows with frozen semen thawed for various lengths of time. VICTOR HURST AND J. W. KELLY, *South Carolina Agricultural Experiment Station.*

P71. Some aspects of the role of glycerol in the metabolism of bovine semen. R. J. FLIPSE AND A. A. BENSON, *Pennsylvania Agricultural Experiment Station.*

P72. A comparison of filtered versus nonfiltered bovine semen after extension and low-temperature storage. M. A. BROWN AND J. C. JOHNSON, *A & M College of Texas.*

P73. Alanine-pyruvate conversions in bovine semen. R. J. FLIPSE AND W. R. ANDERSON, *Pennsylvania Agricultural Experiment Station.*

P74. Investigation of motility of sperm thawed at different temperatures and time intervals. H. H. BRUGMAN, M. E. POORE, AND R. P. SCHMIDT, *University of Maine.*

P75. The recovery of sperm from the exteriorized bovine uterine cervix. H. D. HAFS, D. T. ARMSTRONG, AND R. W. BRATTON, *Cornell University.*

10:15 A.M.–12:30 P.M.

GENERAL BUSINESS SESSION OF THE ASSOCIATION

D. V. JOSEPHSON, *Chairman*
William Neal Reynolds Coliseum

2:00–5:00 P.M.

JOINT WITH PRODUCTION SECTION AND AMERICAN GRASSLAND COUNCIL

Symposium: Forage Quality and Its Evaluation

S. P. MARSHALL, *Chairman*
Williams Hall Auditorium

History, function, and aims of the American Grassland Council. W. A. KING, *The Clemson Agricultural College.*

Effect of soil fertility upon the yield and nutritive value of forages. G. M. WARD, *Kansas State College.*

Effect of growth stage, chemical composition, and physical properties upon the nutritive value of forages. J. T. REID, *Cornell University.*

DISCUSSION

Conditions influencing forage acceptability and rate of intake. M. E. MCCULLOUGH, *Georgia Experiment Station.*

An extension worker's viewpoint on evaluating the nutritive quality of forages. G. W. WERNER, *University of Wisconsin.*

DISCUSSION

2:00–5:00 P.M.

SECTION B. **Reproduction and Genetics**

N. L. JACOBSON, *Chairman*
Riddick Auditorium, Room 242

P76. Effects of an intrauterine infusion of penicillin and streptomycin on reproductive performance in dairy cows. J. M. BUCKALEW AND J. A. ALFORD, *Mississippi State College.*

P77. Effects of oxytocin on bovine ovarian function. D. T. ARMSTRONG AND WILLIAM HANSEL, *Cornell University.*

P78. Morphogenesis and histochemistry of the bovine placenta between 16 and 33 days of gestation. J. S. GREENSTEIN, R. W. MURRAY, AND R. C. FOLEY, *University of Massachusetts.*

P79. The survival of rabbit ova stored at −79° C. MANSUR FERDOWS, C. L. MOORE, AND A. E. DRACY, *South Dakota Agricultural Experiment Station.*

P80. The relationships among duration of estrus, ovulation time, time of service, and fertility of dairy cattle in Louisiana. J. G. HALL, C. BRANTON, E. J. STONE, AND R. B. LANK, *Louisiana State University.*

P81. Bacterial activity of inflammatory exudates from rabbit uteri. H. W. HAWK AND J. F. SYKES, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

P82. Effect of synthalin A on renal function in dairy bull calves. R. R. Anderson and J. P. Mixner, *New Jersey Agricultural Experiment Station.*

P83. Relationships between plasma protein-bound iodine and certain measures of reproductive and lactational performance in dairy cattle. H. D. Lennon, Jr. and J. P. Mixner, *New Jersey Agricultural Experiment Station.*

P84. The effect of pregnant mare serum on the sexually immature bovine testis. Enrique Santamarina and R. P. Reece, *New Jersey Agricultural Experiment Station.*

P85. Some causes of variation in estrual cycle length in Holstein-Friesian heifers. S. E. Mares, A. C. Menge, W. J. Tyler, and L. E. Casida, *University of Wisconsin.*

P86. Age at puberty in Holstein-Friesian heifers. A. C. Menge, S. E. Mares, and L. E. Casida, *University of Wisconsin.*

P87. Genetic improvement of dairy cattle attributable to sires used in artificial insemination in North Carolina. W. L. Tucker and B. R. Farthing, *North Carolina State College.*

P88. Withdrawn.

P89. Heritability and repeatability of some blood constituents of dairy cattle. Mogens Plum and A. B. Schultze, *University of Nebraska.*

2:00–5:00 P.M.

Joint with Manufacturing and Extension Sections

Panel on Education: The Humanistic and Social Studies in a Technical or Scientific Curriculum

William Neal Reynolds Coliseum

5:00–6:30 P.M.

Student Affiliates and Faculty Advisors' Meeting

Williams Hall Auditorium

5:00– 6:00 P.M.

Joint with Extension Section

Meeting of Superintendents of Official Testing

(See Extension Section)

Broughton Auditorium, Room 111

Thursday, June 19

8:00 A.M.–12:00

Section A. **Calf Nutrition and Physiology**

S. P. Marshall, *Chairman*

Riddick Auditorium, Room 242

P90. A comparison of a commercial milk replacer ration with whole milk in a calf-raising system. M. W. NEATHERY, M. E. MCCULLOUGH, O. E. SELL, AND L. R. SISK, *Georgia Mountain Experiment Station.*

P91. A comparison of alfalfa hay, alfalfa silage, and corn silage as sole roughages for young dairy calves. K. E. GARDNER, *University of Illinois.*

P92. The utilization of several fats by dairy calves. D. T. HOPKINS, R. G. WARNER, AND J. I. LOOSLI, *Cornell University.*

P93. Tetra alkylammonium stearate in a milk replacer and a dry calf starter. W. A. OLSON AND J. B. WILLIAMS, *University of Minnesota.*

P94. Effects of feeding volatile fatty acids to young calves. W. G. MARTIN, H. A. RAMSEY, G. MATRONE, AND G. H. WISE, *North Carolina State* College.

P95. The effects of acetate supplementation in calf feeding. J. R. STAUBUS, R. E. BROWN, C. L. DAVIS, AND W. O. NELSON, *University of Illinois.*

P96. Effect of a mixture of chlortetracycline, bacitracin, and penicillin on dairy calves. L. L. RUSOFF AND W. J. MILSTEAD, *Louisiana Agricultural Experiment Station.*

P97. The utilization of urea by young dairy calves as affected by aureomycin. L. D. BROWN, D. R. JACOBSON, J. P. EVERETT, D. M. SEATH, AND J. W. RUST, *University of Kentucky.*

P98. Digestive enzyme activity in the young calf. J. T. HUBER, P. A. HARTMAN, N. L. JACOBSON, AND R. S. ALLEN, *Iowa State College.*

P99. The effects of the duration of whole milk feeding on the growth of dairy calves. R. A. ACKERMAN, R. L. STEELE, AND H. O. HENDERSON, *West Virginia University.*

P100. The value of pasture for young dairy calves. M. C. STILLIONS, C. H. NOLLER, AND B. W. CROWL, *Purdue University.*

P101. The development of rumination in the young calf. E. W. SWANSON AND J. D. HARRIS, JR., *University of Tennessee.*

P102. Magnesium ammonium phosphate precipitated in rumen fluid and the relationship to frothy bloat. R. A. PHELPS, C. K. SMITH, AND C. F. HUFFMAN, *Michigan State University.*

P103. The effect of a scabrous and a nonscabrous diet on rumen motility and intrarumen pressure. H. W. COLVIN, JR., S. D. MUSGRAVE, AND G. F. WILLIAMS, *Oklahoma State University.*

P104. Effects of constant environmental temperature (50 and 80° F.) on growth responses of Brown Swiss, Holstein, and Jersey calves. H. D. JOHNSON, C. S. CHENG, AND A. C. RAGSDALE, *University of Missouri.*

P105. Effect of rising environmental temperatures (35–95° F.) on thyroid I^{131} activity of Holstein, Brown Swiss, and Jersey heifers. H. D. JOHNSON, *University of Missouri.*

P106. Sex differences in certain bovine blood characters. A. B. SCHULTZE, M. PLUM, AND M. G. A. RUMMERY, *University of Nebraska.*

P107. Studies of growth in Holstein, Ayrshire, Guernsey, and Jersey females as determined by: (1) comparison of measurements at several ages to birth measurements as an index. H. P. DAVIS AND I. L. HATHAWAY, *University of Nebraska.*

8:00 A.M.–12:00

SECTION B. **Genetics**
N. L. JACOBSON, *Chairman*
Withers Auditorium, Room 118–119

P108. The heritability of gestation length in dairy cattle. J. C. DEFRIES, R. W. TOUCHBERRY, AND R. L. HAYS, *University of Illinois.*

P109. Relative merits of five ways of adjusting for the herd influence on artificially sired (AI) daughters' records. S. N. GAUNT, *University of Massachusetts.*

P110. Preliminary repeatability estimates of per cent total protein and other constituents in milk. S. D. MUSGRAVE, R. E. WALTON, F. A. GRAYBILL, R. MACVICAR, AND J. B. MICKLE, *Oklahoma State University.*

P111. Error in estimation of lactational yields of solids-not-fat in milk from sampling one day a month. N. R. THOMPSON AND G. C. GRAF, *Virginia Polytechnic Institute.*

P112. Genetic variation in clinical mastitis. C. W. YOUNG, J. E. LEGATES, AND J. G. LECCE, *North Carolina State College.*

P113. Heritability and repeatability of milk yield records of Holstein-Friesian cows in Louisiana with and without certain environmental corrections. C. BRANTON, E. J. STONE, AND G. D. MILLER, *Louisiana State University.*

P114. Estimates of environmental trends and biases resulting from errors in age factors and repeatability. C. R. HENDERSON, *Cornell University.*

P115. Analyses of feed efficiency on six sire groups in the Beltsville Holstein herd. N. W. HOOVEN, JR. AND C. A. MATTHEWS, *Dairy Cattle Research Branch, USDA, Beltsville, Maryland.*

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P116. Relations between weight at first calving and milk production during the first lactation. R. H. MILLER AND L. D. MCGILLIARD, *Michigan State University.*

P117. The relation between weight and production in dairy cattle. B. R. FARTHING AND J. E. LEGATES, *North Carolina State College.*

P118. Relating age conversion factors to herd level. S. R. SEARLE AND C. R. HENDERSON, *Cornell University.*

P119. The effect of season, age, and stage of lactation on type ratings of Holstein cows. C. J. WILCOX, K. O. PFAU, R. E. MATHER, AND J. W. BARTLETT, *New Jersey Agricultural Experiment Station.*

P120. The association of certain type evaluations and type scores of first-lactation Holstein females. E. L. AKINS, T. M. LUDWICK, H. E. RICKARD, E. R. RADER, AND W. M. ETGEN, *The Ohio Agricultural Experiment Station.*

P121. The heritability of various characteristics of body conformation and their association with production of Holstein-Friesian cows in Canada. D. G. GRIEVE, J. C. RENNIE, AND G. E. RAITHBY, *Ontario Agricultural College.*

P122. The contribution of breed of sire, breed of dam, and system of mating to rate of growth in crossbred as compared with purebred dairy cattle. D. C. SHREFFLER AND R. W. TOUCHBERRY, *University of Illinois.*

P123. The effect of two systems of mating on butterfat production of six lines of Holstein-Friesian cattle. C. E. KRAEMER, W. J. TYLER, AND A. B. CHAPMAN, *University of Wisconsin.*

P124. Performance of artificially sired daughters and their contemporary naturally sired herd mates in stratified levels of herd production. N. J. GUDERYON, E. L. CORLEY, AND J. W. DUCKWALL, *University of Wisconsin.*

P125. A method of determining adequate sample size to estimate heritability or repeatability from the analysis of variance. STANLEY WEARDEN, *Kansas Agricultural Experiment Station.*

P126. The genetic structure of dairy cattle herds in terms of additive and dominance relationships. A. E. FREEMAN AND C. R. HENDERSON, *Cornell University.*

8:30–11:30 A.M.

JOINT WITH EXTENSION SECTION AND AMERICAN GRASSLAND COUNCIL

Symposium: Forage Utilization

(SEE EXTENSION SECTION)

Williams Hall Auditorium

EXTENSION SECTION

Tuesday, June 17

9:00–10:00 A.M.

Dairy Records. Increasing Volume of Testing Through Greater Efficiency

D. E. VOELKER, *Chairman*

Broughton Auditorium, Room 111

E1. A device to pipette milk directly into test bottles. W. R. DAVEY, *Pennsylvania State University.*

E2. Results of accuracy studies on weighing and sampling devices. M. E. SENGER, *North Carolina State College.*

10:00 A.M.–12:00

JOINT WITH PRODUCTION SECTION

Panel—What Dairy Records Should Contain to Be of Most Value to Dairymen

GENE MEYER, *Moderator and Discussion Leader*

RALPH WAYNE, *University of Minnesota*

RAYMOND ALBRECTSEN, *Cornell University*

E. E. HEIZER, *University of Wisconsin*

Broughton Auditorium, Room 111

1:30–3:30 P.M.

JOINT WITH MANUFACTURING AND PRODUCTION SECTIONS

Symposium: Use and Control of Pesticides and Antibiotics in Milk Production and Manufacturing: Federal Regulations

(SEE MANUFACTURING SECTION)

Williams Hall Auditorium

1:30–3:30 P.M.

J. D. GEORGE, *Chairman*

Teaching Methods

Stimulating positive thinking. E. A. FAILS, *North Carolina State College.*

Presentation and Discussion of Exhibits

Broughton Auditorium, Room 111

3:30–4:30 P.M.

Extension Section Business Meeting

L. R. FRYMAN, *Chairman*

Report of the Dairy Products Committee

D. E. VOELKER, *Chairman*

Report of the 4-H Club Committee
W. N. PATTERSON, *Chairman*

Report of the Resolutions Committee
CLARENCE PARSONS, *Chairman*

Report of Teaching Methods Committee
J. D. GEORGE, *Chairman*

Report of Nominating Committee
Broughton Auditorium, Room 111

4:30–5:30 P.M.

JOINT PRODUCTION AND EXTENSION BUSINESS MEETING
Joint Committee Reports
L. R. FRYMAN, *Chairman*
Riddick Auditorium, Room 242
Breeds relation. K. A. HUSTON, *Kansas*
Dairy cattle breeding. S. N. GAUNT, *Massachusetts*
Dairy cattle type. J. W. POU, *North Carolina*
Dairy cattle health. J. C. SHAW, *Maryland*

Wednesday, June 18

8:00–10:00 A.M.

Dairy Records and Herd Management
C. W. NIBLER, *Chairman*
Broughton Auditorium, Room 111

E3. Motivation of supervisors and dairymen in identifying cows in dairy herd improvement associations. L. H. RICH AND B. H. CRANDALL, *Utah State University.*

E4. A comparison of machine-calculated records and lactations submitted by DHIA fieldmen. A. G. SENDELBACH AND E. L. CORLEY, *University of Wisconsin.*

E5. Comparison of age conversion factors for Ayrshire butterfat records. R. D. CASSELL, R. S. DUNBAR, JR., R. G. MITCHELL, AND I. D. PORTERFIELD, *West Virginia University.*

E6. Increasing efficiency on farms producing milk for manufacturing purposes. G. S. PARSONS, *North Carolina State College.*

E7. Managing large herds of 100 cows or more. W. R. VAN SANT, *University of Arizona.*

10:15 A.M.–12:30 P.M.

General Business Session of the Association
William Neal Reynolds Coliseum

2:00–4:00 P.M.

4-H Program and Older Youth Program
L. A. JOHNSON, *Chairman*
Broughton Auditorium, Room 111

Swap-Shop of Ideas on Putting 4-H Dairy Programs Across
A panel of state extension dairymen representing the four extension regions. R. E. BURLESON, *Federal Extension Service, USDA, Moderator.*

Older youth and the 4-H Dairy Program. C. C. OLSON, *North Dakota Agricultural College.*

2:00–5:00 P.M.

JOINT WITH PRODUCTION SECTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Quality and Its Evaluation
(SEE PRODUCTION SECTION)
Williams Hall Auditorium

JOINT WITH PRODUCTION AND MANUFACTURING SECTIONS—PANEL ON EDUCATION: THE HUMANISTIC AND SOCIAL STUDIES IN A TECHNICAL OR SCIENTIFIC CURRICULUM

5:00 P.M.

JOINT WITH PRODUCTION
Meeting of Superintendents of Official Testing
K. A. HUSTON, *Chairman*
Broughton Auditorium, Room 111

Thursday, June 19

8:30–11:30 A.M.

JOINT WITH PRODUCTION AND AMERICAN GRASSLAND COUNCIL
Symposium: Forage Utilization
L. R. FRYMAN, *Chairman*
Williams Hall Auditorium

8:30–10:00 A.M.

Panel: Effectiveness of Various Systems of Forage Utilization in Several Regions. J. H. MEYER, *University of California, Moderator*
West—G. Q. BATEMAN, *Utah State University*
Midwest—H. J. LARSEN, *University of Wisconsin*
South—E. J. STONE, *Louisiana State University*
East—W. K. KENNEDY, *Cornell University*
Summary and discussion—J. H. MEYER, *University of California*

10:00–10:05 A.M.

Break

10:05–11:30 A.M.

Economics of various methods of harvesting and utilizing forages. C. R. HOGLUND, *Michigan State University.*

Discussion

Can dairy cattle be bred for increased forage consumption and efficiency of utilization? R. E. MATHER, *Rutgers University.*

Discussion

COMPOSITION AND NUTRITIVE VALUE OF CHEESE PRODUCED FROM MILK TREATED WITH HYDROGEN PEROXIDE AND CATALASE[1]

L. J. TEPLY, P. H. DERSE, AND W. V. PRICE

The Wisconsin Alumni Research Foundation and the Department of Dairy Industry, University of Wisconsin, Madison

SUMMARY

This study provides information on the nutritional value and wholesomeness of cheese made from milk treated with hydrogen peroxide at 5, 10, and 25 times the anticipated normal practical levels. The milks and their wheys obtained in preparing the cheese samples also were tested. The samples were analyzed for proximate composition and for the vitamins, niacin, thiamine, riboflavin, vitamin B_6, pantothenic acid, folic acid, vitamin B_{12}, vitamin A, and beta-carotene. The amino acids, methionine, cystine, tryptophane, and lysine, also were determined. Biological protein evaluation was done for milk, whey, and cheese by feeding these products to rats to provide 9% protein (dry-weight basis) as the sole source of protein in an otherwise complete ration. Additional rat-feeding tests were done on cheese which furnished 14% protein in the diet. The results indicated no marked changes in the composition or nutritive value of milk treated with 0.1, 0.2, and 0.5% hydrogen peroxide, or of the whey or cheese obtained from that milk.

Various dairy products have been treated with hydrogen peroxide for many years and the literature on this subject has been reviewed recently *(7)*. It has been reported that treatment of milk with low levels of hydrogen peroxide for short periods of time (with subsequent enzymatic removal of residual hydrogen peroxide) enables production of cheese of superior quality *(1, 9)*. The beneficial effects of the peroxide appear to be owing to a selective reduction in the population of certain bacteria.

This study was initiated to provide information on the effect of treatment of milk with hydrogen peroxide on the composition and nutritive value of cheese made from that milk. Analysis for proximate composition, vitamins, and amino acids, and biological protein evaluation (rat-feeding) tests were carried out. The cheese samples were made from raw, pasteurized, and 120° F.–treated milks (no peroxide treatment), and from pasteurized and 120° F. milks processed with 5, 10, and 25 times the anticipated normal practice levels of hydrogen peroxide and for approximately 25 times the anticipated treatment period, considering the temperature employed during treatment. The milks themselves, and the wheys obtained from the making of the cheese samples, also were subjected to analysis and biological protein evaluation. Electrophoretic studies were carried out on proteins obtained from the cheese samples.

Received for publication August 13, 1957.

[1] This work was supported by a financial grant from the National Cheese Institute, Chicago, Illinois. The N.C.I. also made arrangements for carrying out the electrophoretic studies.

MATERIALS AND METHODS

Preparation of test materials. The facilities of the University of Wisconsin at Babcock Hall were used for the cheese-making and preparation and packaging of samples. All analyses were carried out promptly on samples held under refrigeration. Samples for rat-feeding tests were stored in a freezer.

The milk used in these experiments was taken from the mixed daily supply from 25 herds. The milk received on October 22, 1956, was clarified, cooled, and treated the next morning.

Cheese was made from raw milk, from milk heated to 120° F., and from pasteurized milk. Portions of the 120° F. and pasteurized milks were further subjected to hydrogen-peroxide-catalase treatments. In all cases, the peroxide treatment was carried out at 120° F. The various portions of milk, each of which weighed 480 lb., were handled as follows: One portion of the mixed raw milk was placed in a 60-gal. cheese vat for manufacturing. Approximately 2,000 lb. of milk was heated to 120° F. in a plate-type heater and run into a 300-gal. tank. One portion of this hot milk was immediately cooled from 120 to 88° F., for cheese-making.

The remainder of the 120°–heated milk in the tank was divided into three 480-lb. portions. To one was added 0.1% hydrogen peroxide, to the second 0.2%, and to the third portion, 0.5%. These percentages represent 100% H_2O_2. (For actual amounts of 35% H_2O_2 used to provide these levels of peroxide, see Table 1.) After holding 10 min. under continuous agitation, each portion

TABLE 1

Amounts of hydrogen peroxide and catalase used in 480-lb. vat-lots of milk

Level of peroxide	H_2O_2 required	35% Peroxide used [a]	Catalase used [b]
(%)	*(lb.)*	*(lb.)*	*(ml.)*
0.5	2.40	6.85	44
0.2	0.96	2.74	17
0.1	0.48	1.37	9

[a] The required amount of 35% hydrogen peroxide was mixed with an equal weight of cold water before mixing with the milk.

[b] The required amount of catalase preparation was mixed with five times its volume of cold water before mixing with the milk.

was cooled from 120 to approximately 90° F. and the catalase preparation was added to destroy the remaining hydrogen peroxide. Each lot of milk was then adjusted to 88° F. for cheese-making.

A second 2,000-lb. portion of the mixed milk was heated in a high temperature, short-time (HTST) pasteurizer to 162° F. for 20 sec., cooled to 110° F., and delivered into a 300-gal. tank, from which one portion was drawn and cooled promptly to 88° F. for cheese-making.

The pasteurized milk remaining in the tank was warmed to 120° F. and divided into three 480-lb. portions. The peroxide treatment of these portions was carried out as just described for the 120° F. milk.

After the pasteurized milk had been divided for the peroxide treatments, an

error in handling made it necessary to discard one of the 480-lb. portions. An equal amount of milk pasteurized by the same method had to be substituted for it. This substitute lot contained a small portion of the milk delivered to the manufacturing laboratory on the day of the experiment. This substitute portion of pasteurized milk was the one treated with 0.2% of hydrogen peroxide.

The hydrogen peroxide used in these experiments was a 35% solution of H_2O_2 designed for use in food processing. The peroxide solution was diluted with an equal weight of cold water before it was added to the milk. The catalase preparation used in these experiments was a stable, buffered solution, at neutral pH, which had been standardized to a potency of 100 Keil Units per cubic centimeter. One Keil unit of catalase is defined as the quantity of catalase required to decompose 1 g. of 100% hydrogen peroxide in 10 min. at 25° C. in an inert atmosphere of CO_2 or N_2. Four times the required theoretical amount of catalase was used to remove all traces of hydrogen peroxide from the milk within 10 min. The catalase preparation was diluted with five times its volume of cold water, then added slowly to the milk, with constant agitation to prevent excessive foaming. The specific amounts used for each concentration of hydrogen peroxide are shown (Table 1). All the peroxide in the treated milk was removed by the catalase before starting cheese-making. The milk was tested for the presence of hydrogen peroxide by adding several drops of a fresh 25% solution of potassium iodide with 2% starch to 5 ml. of milk in a tube. A control tube of identical milk not treated with the hydrogen peroxide was prepared similarly. Complete destruction of hydrogen peroxide was indicated by identical freedom from discoloration in both tubes.

The whey for analysis was removed from each experimental vat just before draining; it was promptly cooled and held at 36° F. To provide whey protein for biological evaluation the following procedure was used:

Unseparated whey removed from the cheese vats at the time of dipping was heated to 200° F. The whey proteins were precipitated by adding to each 100 lb. of the hot whey, 33 ml. of glacial acid diluted with water to 100 ml. The whey-protein precipitate was drained at 70° F. on "Viskon," thoroughly mixed for uniformity, packed in heavily paraffined cottage cheese cartons, fast-frozen at −25° F., and stored at from −10 to −20° F.

All lots of cheese were formed into 5-lb. loaves, wrapped in "Parakote," and held in fiberboard cartons.

The time schedule and method of making the milk into cheese followed procedures which have been described elsewhere *(16)* (Table 2). The time intervals planned for these making procedures required a pH of 6.1 in the curd, at 2.5 hr. after adding rennet to the milk, and a pH of 5.5 at milling, at 4.5 hr. after renneting. When time and acidity measurements did not fit the ideal schedule exactly, then the acidity attained determined the time of dipping or milling. Measurements of pH of curd were made with a Leeds-Northrup portable potentiometer, quinhydrone electrode, and saturated calomel half-cell *(16)*.

Preliminary experiments had shown the desirability of increasing the amount of rennet used in making the cheese from milk treated with these high levels

TABLE 2

Times required for critical stages in the making operation

Milk treatment	Setting[a] to dipping[b]	Dipping to milling[c]	Setting to milling
	hr:min.	hr:min.	hr:min.
Raw milk	2:50	2:30	5:20
Pasteurized			
0.5% H_2O_2	3:08	2:25	5:33
0.2% H_2O_2[d]	3:00	2:16	5:16
0.1% H_2O_2	2:45	2:03	4:48
0% H_2O_2	2:46	1:40	4:26
Heated to 120° F.			
0.5% H_2O_2	3:35	2:10	5:45
0.2% H_2O_2	3:25	2:00	5:25
0.1% H_2O_2	3:21	2:25	5:46
0% H_2O_2	2:45	2:38	5:23

[a] Setting (adding of rennet) occurred when the Marschall rennet test showed ¼-space-change in 15 min.

[b] Dipping (end of running whey) was at pH 6.1.

[c] Milling was at pH 5.5.

[d] This vat-lot of milk was not identical to the others in this series.

of hydrogen peroxide. Therefore, 4 oz. of rennet per 1,000 lb. of milk was used in all lots of milk treated with hydrogen peroxide; 3 oz. per 1,000 lb. was used in the other lots. Under these conditions, milk coagulation was normal.

Development of acidity was slightly slower at first, in vats to which the peroxide had been added. This was especially noticeable in those lots made with 120°–heated milk. This effect might have been prevented by prolonging the period between adding starter and the setting of the vat.

Curd observed at all stages of making was slightly lacking in firmness in those lots which had received 0.5% of hydrogen peroxide. This tendency was not detected in other vat-lots.

For the later feeding of cheese to rats, at a level sufficient to furnish 14% protein in the diet, additional cheese samples were made from pasteurized milk according to the general procedures described above, except that only one level of hydrogen peroxide (0.5%) was used, and the treatment was at 120° for 10 and 30 min. All of the milks were held for an equal period at 120° F. Duplicate sets of samples were made on two separate days and from two different batches of milk.

Analytical methods. The analytical methods employed were, for the most part, well-standardized procedures: moisture *(2)*, ash *(2)*, fat *(2)*, protein *(2)*, carbohydrate (by difference), niacin *(10)*, thiamine *(16)*, riboflavin *(11)*, vitamin B_6 *(1)*, pantothenic acid *(14)*, folic acid *(2)*, vitamin B_{12} *(11)*, vitamin A and beta-carotene *(6)*, methionine, cystine, tryptophane, and lysine *(5)*. For the cheese samples, special methods were used for determining vitamin A and beta-carotene *(2, 6)*, moisture *(13)*, and fat *(12)*. The latter was determined by a modified Babcock test procedure. Hartman *et al. (4)* have demonstrated that cheese requires special treatment for vitamin B_{12} assay; the cheese samples were extracted with acetone and the extracts were then carried through the regular assay procedure.

Biological protein evaluation. Weanling (21-day-old) male albino rats of the Sprague-Dawley strain, weighing 50-60 g., were divided into groups of ten rats each, in such a way that the average weights of all the groups were essentially the same. The animals were housed in individual screen-bottom cages in air-conditioned, temperature-controlled animal quarters; they were supplied with water and test ration ad libitum.

Each sample was incorporated into a semisynthetic rat basal ration as the sole protein source, at such a level as to supply 9.09% protein ($N \times 6.25$) in the finished ration (dry basis). The rations were equalized with respect to fat, ash, carbohydrate, and moisture content, and supplemented with a synthetic vitamin mixture. All of the whey proteins tested contained approximately the following percentages: protein 13, moisture 75, fat 7, ash 0.7, and carbohydrate 4. The casein standard contained 91.3% protein. The final rations for the milk groups contained per 100 g. of solids, 260 g. of moisture; for the whey groups, 60 g., and for the cheese groups, 20 g. In all other respects the rations were identical. The cheese, whey protein, and milk samples were stored frozen. Rations were made up weekly and held in a refrigerator.

The test animals were weighed at weekly intervals through the 6 wk. test period and were fed weighed amounts of test ration. Uneaten rations removed from the cages were collected, combined and, at intervals analyzed for protein and solids content, so as to obtain an accurate estimation of the total food consumption and protein consumption of the animals.

Diet

	(%)
Protein (from sample)	9.09
Corn oil (plus sample fat)	12
Salts I (USP IV) (plus sample ash)	4
Vitamin-glucose mixture[a]	1
Sucrose to	100

[a] Each 100-g. ration provided U.S.P. units: vitamin D, 200, vitamin E, 101, vitamin A, 2,000. Milligrams: choline, 100, inositol, 10, niacin, 4, calcium pantothenate, 4, vitamin B_{12}, 0.003, riboflavin, 0.8, thiamine, 0.5, vitamin B_6, 0.5, menadione, 0.5, folic acid, 0.2, and biotin, 0.04; *para*-aminobenzoic acid, 10 grams.

Electrophoretic studies. The electrophoretic studies were carried out at the National Dairy Research Laboratories, Inc., Oakdale, L. I., New York.

Two grams of cheese were weighed out and triturated with ether until the major portion of the fat was removed. The cheese samples were put into solution in sodium cacodylate–sodium chloride buffer (NaCaCO, 02M; NaCl, 0.08M), 0.1 μ, pH 6.63. An excess of disodium dihydrogen versenate was added and the pH was adjusted to 7.3 with 0.1 N NaOH for maximum sequestering of the calcium present. All the protein went into solution, but residual fat caused turbidity. The solutions were ether-extracted at room temperature until sufficiently clear for analysis. The samples were dialyzed against three changes

of buffer for three days at a temperature of 4° C. Analyses were made at a temperature of 1° C. in a Tiselius-type electrophoresis apparatus manufactured by Frank Pearson Associates. Areas of the components were measured by planimeter, and mobilities were calculated from the descending boundaries. Measurements for mobility were made from the intial boundary to the ordinate bisecting the area of a component.

Feeding tests at a 14% protein level. Feeding tests were carried out with newly prepared samples of cheese which had been treated for 10 and 30 min. with 0.5% H_2O_2. The tests were carried out in the same way as the protein evaluation tests, except for the higher level of sample, to furnish a 14% level of protein; the total fat level was 18%.

RESULTS AND DISCUSSION

Analysis. The analytical results on the milk, cheese, and whey samples are presented (Tables 3, 4, and 5). For the most part, the values fall into the ranges one might expect from data available in the literature. However, in the case of certain nutrients, such as folic acid, which are present at rather low levels in relation to the sensitivity of the test methods, the variation in values among the different milk, cheese, and whey samples is somewhat greater than one would expect from normal assay variation. The microbiological assay procedures employed for the amino acid determinations and most of the vitamin assays are ordinarily considered to give results reproducible, on the average, within a range of ± 10%. For some vitamins, such as folic acid, one expects somewhat greater variation in assay results.

In general, there is no effect of peroxide treatment on levels of nutrients. However, in the case of methionine and lysine, treatment of milk with 0.5% H_2O_2 resulted in a decrease of the levels of these amino acids in cheese produced from this milk, to the extent of 10–25% (dry weight basis). This effect appears to be an indirect one, as it was not noted in the milk and whey samples.

These results substantiate other data which have suggested that, aside from ascorbic acid, the nutrients in milk are rather insensitive to treatment with hydrogen peroxide. Vitamin A in milk has been reported to be relatively stable to peroxide treatment *(14)* and Nambudripad *et al. (10)* found thiamine, riboflavin, niacin, and vitamin B_{12} to be unaffected by addition of 0.03% H_2O_2 (by weight) and by allowing the milk to stand for from 1 to 2 hr. before pasteurizing at 145° F.

Although strong treatment with hydrogen peroxide will produce chemical changes in proteins and amino acids of milk, relatively mild treatment is reported to have little effect *(8, 17)*. This is borne out by the results of the amino acid assays and the electrophoretic studies reported herein.

Protein evaluation. The rats on the protein test were in good health during the experiment and no abnormalities were found on autopsy at termination. Good protein efficiency values (Table 6) were obtained on the various samples and the variation among samples was not unusual for biological tests, with one exception: the protein efficiency ratio for the heat-treated 0.5% peroxide cheese

TABLE 3
Analysis of milk

	Raw	Pasteurized (160° F., 20 sec.) ($\%$ H_2O_2)				120° F.-treated ($\%$ H_2O_2)			
		0	0.1	0.2	0.5	0	0.1	0.2	0.5
Moisture *(%)*	88.55	88.17	88.65	88.64	88.42	88.70	87.88	88.39	89.79
Ash *(%)*	0.74	0.74	0.74	0.72	0.69	0.73	0.72	0.73	0.72
Fat *(%)*	3.30	3.43	3.32	3.47	3.41	3.26	3.49	3.46	3.10
Protein *(%)*	3.25	3.38	3.32	3.32	3.32	3.19	3.51	3.25	3.25
Carbohydrate *(%)*	4.16	4.28	3.97	3.85	4.16	4.12	4.40	4.17	3.14
Niacin *(mg/100 ml.)*	0.084	0.100	.....	.....	0.084	0.078	.....	.....	0.096
Thiamine *(mg/100 ml.)*	0.044	0.047	.....	.....	0.039	0.036	.....	.....	0.038
Riboflavin *(mg/100 ml.)*	0.16	0.17	.....	.....	0.16	0.17	.....	.....	0.18
Vitamin B_6 *(mg/100 ml.)*	0.075	0.064	0.074	0.076	0.073	0.082	0.074	0.055	0.051
Pantothenic Acid *(mg/100 ml.)*	0.45	0.44	.....	.....	0.45	0.43	.....	.....	0.45
Folic Acid *(µg/100 ml.)*	0.57	0.41	.....	.....	0.46	1.14	.....	.....	0.54
Vitamin B_{12} *(µg/100 ml.)*	0.38	0.34	0.46	0.41	0.43	0.46	0.44	0.37	0.32
Vitamin A *(I.U/100 ml.)*	98.3	91.7	.....	.....	93.3	88.3	.....	.....	95.0
Beta-carotene *(µg/100 ml.)*	25.5	26.0	.....	.....	24.7	26.0	.....	.....	24.5
Methionine *(mg/ml)*	0.75	0.75	.....	.....	0.68	0.78	.....	.....	0.70
Cystine *(mg/ml)*	0.21	0.20	.....	.....	0.19	0.22	.....	.....	0.22
Tryptophane *(mg/ml)*	0.44	0.43	.....	.....	0.40	0.40	.....	.....	0.38
Lysine *(mg/ml)*	2.9	2.9	.....	.....	2.8	2.8	.....	.....	3.0

TABLE 4

Analysis of cheese

	Raw	Pasteurized (160° F., 20 sec.) ($\%$ H_2O_2)				120° F.-treated ($\%$ H_2O_2)			
		0	0.1	0.2	0.5	0	0.1	0.2	0.5
Moisture *(%)*	38.07	37.69	40.66	40.90	41.24	38.22	39.96	39.75	41.55
Ash *(%)*	4.05	3.65	3.41	3.60	3.68	3.26	3.77	3.56	3.69
Fat *(%)*	32.6	32.4	31.0	30.2	31.0	32.3	31.4	30.8	30.3
Protein *(%)*	27.24	26.09	25.07	26.09	25.33	26.54	24.31	25.46	26.16
Niacin *(mg/100 g.)*	0.080	0.086	.....	.....	0.084	0.092	.....	.....	0.070
Thiamin *(mg/100 g.)*	0.097	0.030	.....	.....	0.020	0.023	.....	.....	0.024
Riboflavin *(mg/100 g.)*	0.48	0.43	.....	.....	0.32	0.38	.....	.....	0.38
Vitamin B_6 *(mg/100 g.)*	0.078	0.077	0.086	0.080	0.091	0.098	0.108	0.102	0.109
Pantothenic Acid *(mg/100 g.)*	0.49	0.51	.....	.....	0.52	0.41	.....	.....	0.43
Folic Acid *(mg./100 g.)*	0.038	0.037	.....	.....	0.044	0.033	.....	.....	0.059
Vitamin B_{12} *(µg/100 g.)*	0.54	0.78	0.74	0.62	0.95	1.33	1.54	0.61	0.58
Vitamin A *(I.U/100 g.)*	1,000	1,050	.....	.....	900	1,000	.....	.....	900
Beta-carotene *(µg/100 g.)*	200	220	.....	.....	190	195	.....	.....	175
Methionine *(mg/g)*	7.07	7.40	.....	.....	5.42	6.73	.....	.....	5.22
Cystine *(mg/g)*	1.00	1.00	.....	.....	0.85	1.02	.....	.....	0.88
Tryptophane *(mg/g)*	2.9	5.0	.....	.....	3.0	3.1	.....	.....	3.0
Lysine *(mg/g)*	25.2	25.0	.....	.....	20.9	23.8	.....	.....	22.0

TABLE 5

Analysis of whey

	Raw	Pasteurized (160° F., 20 sec.) ($\% H_2O_2$)				120° F.–treated ($\% H_2O_2$)			
		0	0.1	0.2	0.5	0	0.1	0.2	0.5
Moisture *(%)*	93.40	93.42	93.46	93.36	93.65	93.46	93.54	93.56	93.45
Ash *(%)*	0.56	0.56	0.58	0.57	0.54	0.54	0.57	0.58	0.56
Fat *(%)*	0.34	0.27	0.26	0.32	0.33	0.33	0.33	0.40	0.37
Protein *(%)*	1.02	0.96	0.96	1.02	1.08	1.02	1.02	1.08	1.08
Carbohydrate *(%)*	4.68	4.79	4.74	4.73	4.40	4.65	4.54	4.38	4.54
Niacin *(mg/100 ml.)*	0.060	0.074	.....	.....	0.120	0.074	.....	.....	0.098
Thiamine *(mg/100 ml.)*	0.049	0.066	.....	.....	0.056	0.043	.....	.....	0.043
Riboflavin *(mg/100 ml.)*	0.13	0.12	.....	.....	0.14	0.13	.....	.....	0.13
Vitamin B_6 *(mg/100 ml.)*	0.059	0.058	0.062	0.031	0.061	0.063	0.058	0.061	0.057
Pantothenic Acid *(mg/100 ml.)*	0.35	0.39	.....	.....	0.37	0.39	.....	.....	0.32
Folic Acid *(μg/100 ml.)*	5.2	2.4	.....	.....	4.4	5.0	.....	.....	4.3
Vitamin B_{12} *(μg/100 ml.)*	0.24	0.24	0.28	0.20	0.24	0.25	0.30	0.18	0.21
Methionine *(mg/ml)*	0.17	0.17	.....	.....	0.17	0.17	.....	.....	0.17
Cystine *(mg/ml)*	0.18	0.19	.....	.....	0.15	0.17	.....	.....	0.18
Tryptophane *(mg/ml)*	0.14	0.15	.....	.....	0.14	0.15	.....	.....	0.13
Lysine *(mg/ml)*	0.83	0.83	.....	.....	0.83	0.82	.....	.....	0.89

sample was 1.82, compared to a value of 2.34 for the control. In a repeat test on this sample (which had been stored in a freezer), a protein efficiency ratio of 2.14 was obtained, with a value of 2.40 for the control. The results as a whole indicate little, if any, change in the nutritional value of cheese protein when the milk from which it is made is treated with peroxide as described.

Electrophoresis. The electrophoretic analyses of proteins of cheeses made from milk treated with various levels of H_2O_2 are shown (Table 7 and Figure 1). The cheese samples were stored in the frozen state for approximately 2 mo., then stored at 40° F. for up to 2 wk. before examining. The four samples which had the longest storage periods *(1, 2)* were all low in concentration of beta-casein in the descending patterns, indicating possible storage changes. The descending patterns of the pasteurized series have marked similarity in appearance. Samples 2 and 4, treated, respectively, with the lowest and highest concentration of H_2O_2, show no real differences in their analyses. The control Sample 1 and Sample 3 (0.2% H_2O_2) are both low in beta-casein in the descending pattern. This may be due to the slightly longer storage periods for these samples. The mobility of the alpha-casein complex in the control Sample 1 seems rather low compared to the other pasteurized samples. This difference can not be considered as the result of the peroxide treatment specifically, since Samples 5 (120° F., no H_2O_2) has mobilities similar to the peroxide-treated samples.

In the 120° F. series, no definite changes due to the peroxide treatment were noticed. As in the pasteurized series, the samples with the longest storage periods were low in concentration of beta-casein in the descending patterns. All samples, both pasteurized and 120° F.–treated, showed some dissociation of the alpha-casein complex in the ascending pattern. The degree of dissociation was not related to the strength of the peroxide treatment.

The absence of an effect on electrophoretic patterns, definitely attributable to the peroxide treatments used in these experiments, is what we might expect from electrophoretic studies on peroxide-treated milk reported by Lück and Joubert *(8)*. These workers did observe some changes in the β-lactoglobulin diagrams when skimmilk was treated with 0.25 and 1% H_2O_2 (39%) for from 3 to 5 hr. at room temperature, at which time the catalase was inactivated. After the catalase inactivation, the H_2O_2 level remained constant at from 0.015 to 0.10 *M*. The milk was held for from four to six days after catalase inactivation. Lück and Joubert note that other workers have observed similar effects on the β-lactoglobulin from other treatments, such as radiation with ultraviolet light, or heat treatment.

Feeding tests at a 14% protein level. All rats were in good health during the experiment and no abnormalities were found on autopsy at termination. Analyses of the cheese are given (Table 8) and weight gain and food consumption data also are presented (Table 9). On the average, the animals on the peroxide-treated cheeses gained from 5 to 10% less than the animals on the control cheeses. Statistical analysis showed that variation in feed efficiency among groups was not significantly different (at the 5% level) from variation in feed efficiencies within the groups. There was good correlation between weight gain

TABLE 6

Biological protein evaluation results

(Rat weights are averages for ten rats—in grams)

Milk treatment (% H_2O_2):	Raw	Pasteurized (162° F., 20 sec.)				120° F–treated			
	0.0	0.0	0.1	0.2	0.5	0.0	0.1	0.2	0.5
Milk samples									
Initial weight	53.3	53.6	53.7	52.5	53.2	53.4	52.5	52.3	52.0
6-wk. weight	176.9	185.6	175.6	170.7	173.8	192.9	178.9	185.4	179.5
Food consumption[a]	457.7	132.0	121.9	118.2	120.6	139.5	126.4	133.1	127.5
Protein efficiency[b]	3.04	498.1	479.1	461.2	483.2	514.5	498.0	517.2	489.7
Total gain	123.6	3.00	2.88	2.95	2.94	3.01	2.82	2.97	2.94
Whey protein samples									
Initial weight	52.5	52.7	52.5	52.6	52.7	52.8	52.4	52.3	52.7
6-wk. weight	200.4	206.3	187.0	194.6	183.9	188.0	209.3	189.4	202.2
Total gain	147.9	153.6	134.5	142.0	131.2	135.2	156.9	137.1	149.5
Food consumption[a]	505.9	525.1	474.7	496.8	485.7	490.8	503.3	474.0	488.1
Protein efficiency[b]	3.19	3.19	3.04	3.12	2.92	2.97	3.43	3.16	3.36
Cheese samples									
Initial weight	51.9	52.0	51.3	51.7	52.1	52.5	51.1	52.9	52.9
6-wk. weight	129.6	140.1	140.6	124.4	123.6	136.8	137.4	135.9	111.3
Total gain	77.7	88.1	89.3	72.7	71.5	84.3	86.3	83.0	58.4
Food consumption[a]	361.9	377.5	402.0	352.8	359.7	380.9	378.3	377.6	318.0
Protein efficiency[b]	2.27	2.52	2.42	2.20	2.10	2.34	2.49	2.33	1.82

Casein standards:	A[c]	B[d]		Repeat test on cheese from heat-treated milk	
				H_2O_2	0.5% H_2O_2
Initial weight	52.4	51.8	Initial weight	55.0	54.8
6-wk. weight	145.8	172.3	6-wk. weight	136.8	120.8
Total gain	93.4	120.5	Total gain	81.8	65.2
Food consumption	404.1	443.5	Food consumption	353	323
Protein efficiency	2.33	2.82	Protein efficiency	2.40	2.14

[a] Dry basis.
[b] Grams gain per gram protein consumed.
[c] A = diet moisture content as for whey diet.
[d] B = diet moisture content as for cheese diet.

TABLE 7

Electrophoretic analyses of Cheddar cheese proteins

Sample	Date of analysis	Treatment	Rel. concentration ascending	Rel. concentration descending	Mobility descending
			(%)		(1×10^5 cm²/sec volt)
1	1/24/57	Pasteurized, no H_2O_2	A[a] 31.4	A 17.3	A −1.90
			B[a] 62.2	B 82.7	B −4.50
			C[a] 7.3		
2	1/15/57	Pasteurized, 0.1% H_2O_2	A 37.0	A 28.2	A −2.28
			B 49.7	B 71.8	B −5.23
			C 13.3		
3	2/1/57	Pasteurized, 0.2% H_2O_2	A 24.3	A 18.1	A −1.70
			B 67.3	B 63.8	B −4.41
			C 8.4	C 18.1	C −6.78
4	1/17/57	Pasteurized, 0.5% H_2O_2	A 33.0	A 30.0	A −2.08
			B 51.8	B 70.0	B −5.06
			C 15.2		
5	1/14/57	120° F., no H_2O_2	A 32.9	A 27.0	A −2.32
			B 53.9	B 73.0	B −5.28
			C 13.2		
6	1/22/57	120° F., 0.1% H_2O_2	A 29.8	A 18.0	A −2.25
			B 52.2	B 82.0	B −5.19
			C 18.0		
7	1/21/57	120° F., 0.2% H_2O_2	A 35.3	A 22.4	A −2.20
			B 55.4	B 63.4	B −4.76
			C 9.3	C 14.2	C −6.22
8	1/18/57	120° F., 0.5% H_2O_2	A 33.5	A 30.1	A −2.10
			B 49.1	B 69.9	B −5.06
			C 17.4		

Buffer: Na cacodylate (0.02 *M*) NaCl(0.08*M*), pH 6.63, 0.1 U.
Av. E = 4.90 volts/cm.
Av. T = 15,547 sec.
[a] Components: A = beta-casein, B = alpha-casein complex, C = dissociated fraction from alpha-casein complex.

TABLE 8

Analysis of cheese used in feeding tests at a 14% protein level.

	Protein	Fat	Moisture	Ash
		(%)		
7-16 Control	25.9	31.1	38.4	4.24
7-17 Control	25.1	31.7	37.5	4.86
7-16 0.5% H_2O_2—10 min.	23.5	28.9	42.9	3.95
7-17 0.5% H_2O_2—10 min.	22.8	28.8	42.9	3.64
7-16 0.5% H_2O_2—30 min.	23.0	28.6	43.2	4.01
7-17 0.5% H_2O_2—30 min.	22.0	28.0	43.2	4.36

TABLE 9

Feeding trials with cheese furnishing 14% protein

	Average initial weight	Average weight after 6 wk.	Average weekly weight gain	Average weekly feed consumption
		(g.)		
Control, 7-16	63.2	259.9	32.8	116.8
Control, 7-17	63.1	256.7	32.3	119.3
H_2O_2—10 min., 7-16	63.1	247.8	30.8	116.0
H_2O_2—10 min., 7-17	63.2	241.1	29.6	110.9
H_2O_2—30 min., 7-16	63.1	239.3	29.4	113.3
H_2O_2—30 min., 7-17	63.1	246.2	30.5	119.3

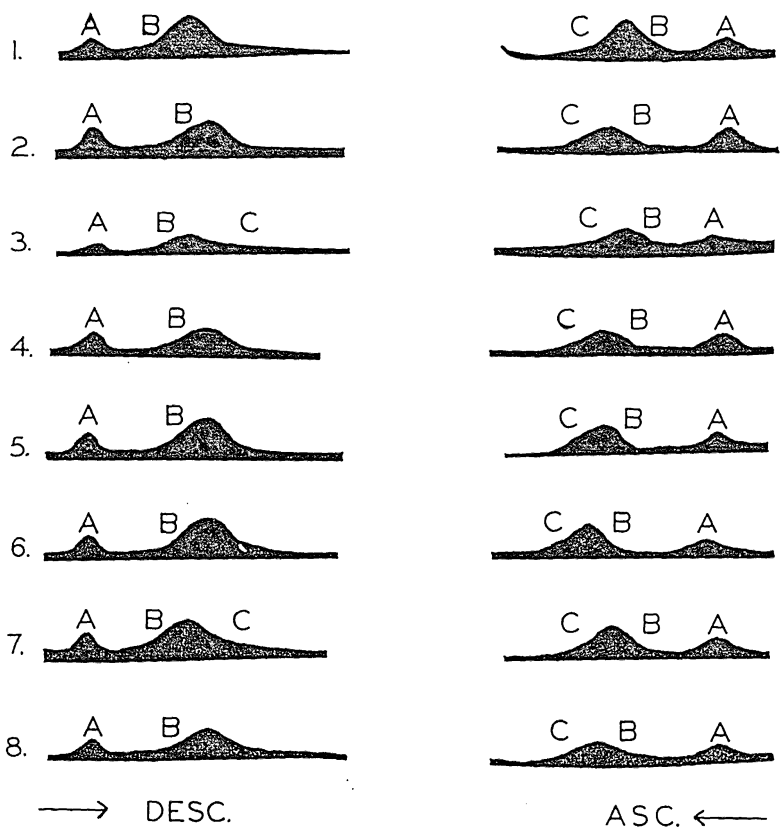


FIG. 1. Electrophoresis of proteins of cheeses made from milks treated with various levels of hydrogen peroxide. The sample treatments are given (Table 7).

and feed consumption, both among groups and within each group. The moderate difference in growth between control and experimental groups was apparently related to a slight difference in feed consumption.

CONCLUSIONS

Analytical values for milk, cheese, and whey samples were in good agreement with published values. In general, there was no effect of peroxide-catalase treatment of milk on levels of nutrients in the milk, or in cheese or whey obtained from that milk. Considering the data on a dry-weight basis, treatment of milk with 0.5% peroxide lowered the values for cystine and methionine in the cheese by from 10 to 25%.

Good protein efficiency ratios were obtained on all of the milk, cheese, and whey samples, and variation was within normal limits with one exception, in the case of the cheese made from 120° F. 0.5% peroxide-treated milk. However, the definite lowering of the protein efficiency ratio indicated in the first test on this sample was not confirmed in a repeat test on this particular sample.

Electrophoretic studies on proteins from the cheese samples showed only

minor differences, which did not appear to be directly related to the peroxide treatment.

The rat-feeding tests carried out on duplicate sets of cheese made from milk treated with 0.5% hydrogen peroxide for 10 and 30 min., and with the cheese furnishing 14% protein diet, showed only a slight difference in growth between control and experimental groups.

The results as a whole indicate no marked changes in the composition or nutritional value of milk treated with 0.1, 0.2, or 0.5% hydrogen peroxide under the conditions described, or in the cheese or whey obtained from that milk.

ACKNOWLEDGMENTS

The manipulations and technical control of the milk treatments and cheese-manufacturing and whey protein–separating operations were simplified by the generous, skillful help of the following men: Vern Adams, R. L. Eldredge, Fred Feutz, Don Otteson, Harold Steinke, L. Thomas, and J. Schlichting of the Cheese Division of the Borden Company; Z. D. Roundy of the Armour Laboratories; and D. B. Emmons and Roy Tjepkema of the University of Wisconsin.

Becco Chemical Division of the Food Machinery and Chemical Corporation, Buffalo, New York, furnished the 35% hydrogen peroxide, and the Armour Laboratories, Kankakee, Illinois, provided the catalase (Armalase A100) used in these experiments.

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DISTRIBUTION OF PROPIONIBACTERIA IN SWISS CHEESE

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SUMMARY

Conventional wheel and rectangular rind-bearing block Swiss cheeses were studied to determine the distribution of propionibacteria. Samples were taken from three predetermined locations in six different Swiss cheeses which were from 1 to 16 mo. old. Propionibacteria were determined by the plating method of Kambar *et al. (3),* and cultures were taken from agar plates and identified. Few propionibacteria were found in samples taken 1/8-in. below the surface of the cheese. With one exception, samples taken 7/8-in. below the surface contained many more propionibacteria than were found in the exterior portions. Proceeding inward to sections 2 5/8 in. below the cheese surface, the numbers increased, though not proportionally. These data suggest that the decrease in numbers of propionibacteria toward the exterior of Swiss cheese is probably the result of such unfavorable conditions as lower moisture, higher salt concentrations, and higher oxidation-reduction potentials.

From a bacteriological aspect, the outstanding characteristic of Swiss cheese is the presence of large numbers of propionic acid bacteria. The role of these organisms in the development of flavor in Swiss cheese has been clearly established by many workers. In numerous instances, the numbers of these organisms in cheese at various stages of the make and curing operation have been determined. No studies have been reported which have shown the distribution of propionibacteria within individual samples of Swiss cheese. The object of this investigation was to determine the influence of the usual make procedures upon the distribution of propionibacteria in Swiss cheese.

EXPERIMENTAL PROCEDURE

Cheeses. Information pertaining to the identification of the cheeses used in this study is shown (Table 1). Cheeses W1 through W4 were conventional wheel-shaped Swiss cheeses obtained through a commercial source. Cheeses B11 and B12 were experimental cheeses which differed from conventional Swiss cheese only in their shape; these were rectangular blocks, each weighing approximately 80 lb. Flavor and eye development in each cheese was considered good by a competent judge.

Sectioning cheese for analysis. A 4-in.-wide cross section was cut vertically through the middle of the cheese; from this section three layers parallel to the top surface were removed for analysis. The first section consisted of a layer 1/4-in. deep taken 1/8-in. under the surface of the cheese. This section hereafter is referred to as Position 1. Then, 1/2-in. of cheese was removed and discarded. A layer 1/4-in. deep was then taken; this section is referred to as Position 2. The next 1 1/2 in. of cheese was removed and discarded. The next layer, 1/4-in.

Received for publication October 8, 1957.

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TABLE 1
Identification and description of experimental Swiss cheeses

Cheese No.	Form	Age
		(mo.)
B11	80-lb. Block	1
B12	80-lb. Block	1
W1	Wheel	2
W2	Wheel	2
W3	Wheel	16
W4	Wheel	16

deep, is referred to as Position 3. An 11-g. sample for plating purposes was taken from that portion of each layer that approximated the center of the cheese as viewed from the surface. Adjacent portions were taken for chemical analysis.

Bacteriological analysis. The cheese used for plating was weighed (11 g.) into a sterile container, then ground in a mortar to a smooth mass. During the grinding, 99 ml. of sterile aqueous 2.0% sodium citrate was added. Grinding was continued until a milky homogenous suspension resulted.

The plating method of Kambar *et al. (3)* was used. Duplicate plates were prepared. After counting, one plate which represented the proper dilution and possessed uniformly distributed bacterial colonies was selected for each cheese sample.

One hundred adjacent colonies from each plate were inoculated into tubes of the broth described by Kambar *et al. (3)* and were incubated at 30° C. After sufficient growth had occurred, the cultures were examined morphologically, using the gram stain. Cultures were tested for catalase production by combining 1 ml. of broth culture with 1 ml. of 3.0% hydrogen peroxide in a clean test tube. Cultures showing any evolution of gas were considered catalase positive. The ability of the cultures to ferment sodium lactate was determined. The medium used in these studies contained 0.5% Difco yeast extract, 2.0% B.B.L. Trypticase, 1.0% sodium lactate, and 1 ml. of a 1.6% solution of brom thymol blue. These ingredients were dissolved in distilled water; the broth was then adjusted to pH 7.0 with sodium hydroxide. Sterilization was accomplished by heating at 121° C. for 20 min. Cultures developing acidity in this medium were considered capable of fermenting sodium lactate. The appearance of the growth in broth developed by each colony was also recorded, as an aid toward classification.

Total aerobic counts were made, using the carrot-liver extract medium of Barber and Frazier *(2)*. This medium was modified slightly. B.B.L. Trypticase was used in place of Difco Neopeptone and the agar content was increased from 1.2 to 1.5%.

Chemical analysis. Standard methods *(1)* for the determination of moisture and salt were used. Samples for the determination of moisture were not preheated on a steam bath. Instead, samples were preheated in an oven without vacuum for 15 min. at 100° C.

RESULTS

The distribution of propionibacteria in Swiss cheese followed a definite pattern (Table 2). In all of the six cheeses examined, the propionibacteria de-

TABLE 2

Bacterial content and composition of six Swiss cheeses

Designation of cheese and sample location	Depth of sample from surface	Propionibacteria[a]	Total aerobic count[a]	Moisture content of cheese sample	Salt content of cheese sample	Salt in moisture
	(in.)	*(per g.)*		*(%)*		
B11–1	⅛	1	35	24.72	2.03	8.21
B11–2	⅞	700	186	36.60	1.27	3.47
B11–3	2⅝	1,350	246	38.77	0.31	0.80
B12–1	⅛	1	35	25.54	2.35	9.20
B12–2	⅞	1,200	151	35.74	1.29	3.61
B12–3	2⅝	2,170	189	38.19	0.23	0.60
W1–1	⅛	1	98	33.23	0.94	2.83
W1–2	⅞	74	216	37.72	0.80	2.12
W1–3	2⅝	390	202	38.73	0.36	0.93
W2–1	⅛	71	193	31.53	1.03	3.27
W2–2	⅞	1	38	37.48	0.95	2.53
W2–3	2⅝	410	228	38.87	0.42	1.08
W3–1	⅛	77	30	29.82	0.53	1.78
W3–2	⅞	440	43	36.05	0.65	1.80
W3–3	2⅝	610	21	37.38	0.69	1.85
W4–1	⅛	2	11	28.81	0.71	2.46
W4–2	⅞	17	33	35.91	0.89	2.48
W4–3	2⅝	107	19	38.39	0.86	2.24

[a] Counts expressed as multiples of one million.

creased in number from the interior portions of the cheese to the exterior. A minor exception to this pattern occurred upon examination of Cheese W2. Position 3 of Cheese W2 contained 410,000,000 propionibacteria per gram. Position 2, which was 1½ in. nearer the surface, contained less than 1,000,000 propionibacteria per gram. Position 1, however, located ½-in. nearer the surface than Position 2, and only ⅛-in. under the surface of the cheese, yielded 71,000,000 propionibacteria per gram.

Propionibacteria in Cheese B11 decreased from 1,350,000,000 per gram in Position 3 to 1,000,000 per gram in Position 1. Cheese B12, which was made on the same day and was cured simultaneously with Cheese B11, contained 2,170,000,000 propionibacteria per gram in the interior of the cheese (Position 3). Position 1 of this same cheese contained less than 1,000,000 propionibacteria per gram. Another example of this decrease in propionibacteria existed in Cheese W1. Position 3 in this instance contained 390,000,000 propionibacteria per gram; in Position 1, less than 1,000,000 per gram were present. Cheeses W3 and W4, which had been aged for 16 mo., again showed this typical distribution of propionibacteria. Cheese W3 contained 610,000,000 propionibacteria per gram at Position 3 in the interior of the cheese; Position 1 of this same cheese yielded 77,000,000 per gram. Cheese W4 at Position 3 had 107,000,000 propionibacteria per gram, whereas Position 1 yielded only 2,000,000 per gram.

As has been shown, propionibacteria are not distributed evenly throughout conventional block or wheel-shaped Swiss cheese. Samples taken 2⅝ in. below the surface of six different Swiss cheeses were, in general, quite high in numbers of propionibacteria. Proceeding outward, the number of propionibacteria decreased strikingly. All of the six samples taken ⅞-in. under the surface contained

fewer propionibacteria than were present in the sample areas closer to the center of the cheeses. The decrease in numbers of these organisms was most noticeable in the comparison between the samples taken 7/8- and 1/8-in. under the cheese surfaces.

Considering other types of bacteria, that is, those capable of growing aerobically on carrot-liver infusion agar, this pattern did not exist. Three of the six cheeses did not show any significant decrease in bacterial population of this type from the interior to the exterior of the cheese. In addition, organisms from the aerobic plates were cultured in litmus milk and were examined microscopically. There was no significant variation in numbers or types of these organisms throughout the six different cheeses. As would be expected, streptococci and lactobacilli predominated.

Results of the chemical analysis of the cheese for moisture and salt percentage were normal. In every instance, the interior portions of the cheese were higher in moisture content than the exterior portions. Considering salt content, the reverse of this was true, depending upon the age of the cheese. With cheese 2 mo. and younger, the salt was concentrated in the outer portions of the cheese. The salt in Samples W3 and W4, 16 mo. old, was distributed evenly throughout the cheese. When expressed as the per cent of salt in the moisture present at the different sample locations, this uniformity of salt distribution throughout Cheeses W3 and W4 becomes even more apparent.

DISCUSSION

The great variation in numbers of propionibacteria at the different sample locations is undoubtedly the result of several inseparable factors. Propionibacteria are characterized by their general inability to grow aerobically unless present in large numbers. During the earlier stages of Swiss cheese making, relatively few propionibacteria are present in the curd. It is during this time that the concentration of salt in the outer portions of the wheel is at its highest level. In addition, the outer portion of the curd mass at this time is low in moisture. High concentrations of salt in the moisture phase would inhibit growth of propionibacteria, especially if the oxidation-reduction potential were unfavorable.

At the same time, conditions at the surface of the cheese are at the most unfavorable, that is, during the early part of the warm-room treatment, conditions in the interior of the cheese would be most favorable for the growth of propionibacteria. Here, the curd would have a low oxidation-reduction potential, as has been determined in this laboratory. Moisture content would be comparatively high and salt content would be low. The temperature at this point is selected to favor the growth of the organisms (72–78° F.). Consequently, under these favorable conditions the propionibacteria in the interior of the cheese are able to increase greatly. At this same time, the same organisms in the exterior portions of the cheese are growing more slowly. This creates the great disparity in the specific bacterial population. After the warm-room treatment, the cheese is held at a temperature range (40–45° F.) not conducive to growth of propioni-

bacteria. As a result, the difference in numbers would have little chance to equilibrate itself.

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INFLUENCE OF PASTEURIZATION AND COOL-AGING ON THE BEHAVIOR OF PURE SALT SOLUTIONS PREPARED IN ACCORDANCE WITH THE COMPOSITION AND CONCENTRATIONS IN MILK

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SUMMARY

A pure salt solution was prepared to simulate the composition of salts in milk serum, in order to study the mineral balance in milk. The salts were distributed into soluble and colloidal phases and a sediment was separated from the solution by centrifugation. Storage resulted in formations that resembled "lime grains." Analysis showed that 35.9, 72.9, 54.8, and 80.9% of Ca, Mg, P, and citric acid, respectively, were soluble. The Ca:P ratio in the sediment was 1.46, indicating predominance of tricalcium phosphate.

Heating decreased soluble Ca and pH, whereas cool-aging had the reverse effect. Variations in soluble Mg and citric acid were insignificant, but soluble P decreased on heating and on cool-aging in the pasteurized lot. The Ca:P ratio in the sediment from the untreated aliquot decreased to 1.26 on cool-aging, but pasteurization increased it to 1.63, which decreased again to 1.08 on cool-aging. Adding Ca increased Ca:P ratio, whereas adding P decreased it. The pH decreased with added Ca and increased with added P. Undissolved Ca, Mg, and citric acid in the sediment, when accounted for as tribasic salts, left 0.5 mg. of P and 0.4 mg. of Mg unaccounted for per 100 ml. of solution.

In attempting to explain the various phenonema resulting in alterations in the ionic equilibria in milk under various processing conditions, resemblance with the behavior of pure salts is often sought and indicated. Such comparisons have been common in explaining: the influence of heat treatments and sterilizatioin on pH of milk; variations in rennet-coagulation time relative to heat treatment and temperature history; effect of addition of salts as correction in evaporated milk, and other phenomena. The alterations in pH of milk on heating have been explained in terms of shifts of tricalcium phosphate equilibrium *(6, 7, 8, 9)*, as is rennet hysteresis, which also is explained, in part, as being related to the ionic equilibria in milk. Although many explanations appear to be reasonable, it is obvious that observations on the mineral balance in milk should be interpreted with a consideration of the proteins, lactose, fat, and other minor constituents present. Studies on simplified systems of various types have been made to determine the extent to which these interpretations are justified, as well as to verify the observations made on milk *(3, 4, 11, 12, 13)*. The present study was undertaken to see if changes in pH and salt balance found under various processing treatments in milk can be observed and verified under similar conditions, in mixtures of pure salts prepared to simulate the composition of salts in milk.

Zoller *(14)* reported studies on salt solution, where he followed the precipi-

Received for publication July 30, 1957.

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tation of calcium quantitatively and measured the variations in pH resulting from heat treatment. He reported a progressive loss of calcium from the solution with increasing intensity of heat. This was also accompanied by a proportionate increase in the hydrogen ion concentration. The data on the composition of the salt solution, and other analytical observations, were not reported.

In the present study, an attempt to arrive at a salt solution was based on the synthetic milk studies of Clark *(1)*. On elimination of proteins and sulfuric acid a mixture was obtained, but the pH was much higher than is usual for normal milk. After several trials, the following combination of salts was found satisfactory:

	(per liter)
$CaCl_2 \cdot 2H_2O$	2.62 g.
MgO	0.31 g.
KH_2PO_4	3.26 g.
Citric Acid $\cdot H_2O$	2.06 g.
NaOH solution (1 *N*)	21.0 ml.
KOH solution (0.97 *N*)	10.0 ml.
$Ca(OH)_2$ (0.1 g. Ca/100 ml.)	150.0 ml.

The expected final composition in grams per liter was: calcium, 0.87, magnesium, 0.19, phosphorus, 0.74, citric acid, 1.88, sodium, 0.48, potassium, 1.32, and chlorine, 1.27.

In arriving at the level of calcium in this solution, the amount of calcium known to be in combination with casein was deducted from the total calcium in milk. In like manner, the amount of phosphorus known to exist in combination with casein also was discounted. Based on the data of Van Slyke and Bosworth *(7)*, 1.63% P_2O_5, and according to de KaKdt and van Minnen *(5)*, 1.5% CaO, were deducted, in arriving at the composition of the solution.

It may be pointed out that the final composition of this salt solution is considerably different from the composition of salts in the synthetic milk of Clark. After discounting the amounts of minerals combined with proteins, as indicated, the composition can be regarded to fall within the normal range for calcium, magnesium, phosphorus, and citric acid in normal milk. Further, the di- and trivalent ions were the primary concern of this study and, since the influence of the monovalent ions is relatively small, these were not controlled to the same extent as the former. This became necessary and incidental to achieving a desirable pH, which was obtained by using NaOH.

The MgO was dissolved in 500 ml. of water. Then the calcium chloride, dissolved in 50 ml. of water, was added and then citric acid. This solution had a pH of 3.6. While stirring with a mechanical stirrer, 10 ml. of KOH, followed by 15 ml. of NaOH, were added. The potassium phosphate was dissolved in 200 ml. of water and added very slowly from a burette (with vigorous stirring). About 150 ml. of calcium hydroxide were then added dropwise and the pH adjusted to 6.7 with an additional 6.0 ml. of NaOH. The solution obtained was made to one liter with water and held about 10 hr. in a refrigerator at

45° F. The pH decreased to 6.67. This solution was used in further study, which is described. Any treatments given were in addition to this holding period of 10 hr. The solution was warmed to room temperature and the actual amounts of calcium, magnesium, phosphorus, and citric acid were determined analytically. The solution showed some evidence of grain formation in an aliquot which was allowed to stand in cold for from three to four days. The grains resembled the typical lime grains that occur in evaporated milk.

A viscous and colloidal sediment separated upon centrifugation of the solution at room temperature for 15 min. at 1,500 r.p.m. The supernatant was analyzed for soluble salts. The analyses revealing the distribution of calcium, magnesium, phosphorus, and citric acid are given (Table 1). The lack of agreement between the expected amounts of calcium, magnesium, phosphorus, and citric acid and the amounts found on actual analyses were owing to approximations involved in weighing and also reflect impurities in the reagents.

The effects of pasteurization and cool-aging on the distribution of these salts into sediment and supernatant also were studied. The temperature of a 250-ml. aliquot of the solution was increased to 143° F. in a water bath with constant agitation and maintained for 30 min. It was then cooled and transferred to a 250-ml. volumetric flask and the volume restored again with distilled water. These results also are presented (Table 1). Results of the influence of added calcium and phosphorus salts under these conditions are given (Table 2). Significant differences were found in the pH of the solution under the various treatments and are reported (Table 3).

DISCUSSION

It was indicated by Sommer and Menos *(8)* that the calcium and phosphorus in milk exceeds the solubility product of tricalcium phosphate. Therefore, milk contains precipitated tricalcium phosphate in a finely divided form. In the pure salt solution, also, the precipitate was found to be colloidal. The colloidal phase, however, did not stay dispersed in the salt mixture, and the proteins in milk maintained the dispersion because of their protective effects.

Tricalcium phosphate precipitation does not occur instantaneously on heating, but several hours are required for equilibrium. This fact was supported by the behavior of the pure salt solution. With a decrease in the amount of soluble

TABLE 1

Influence of heating and cool-aging on the dstiribution of salts in the supernatant and centrifugal sediment and on pH and Ca:P ratios

	Calcium	Magnesium	Phosphorus	Citric Acid	pH	Ca:P ratios in sediment
	(mg/100 ml.)					
1. Total	94.0	19.2	75.5	155.1	6.67	.....
2. Supernatant						
a. In untreated	33.8	14.0	44.1	125.5	6.67	1.46
b. In (a) after 24 hr.	30.0	15.7	37.3	130.6	6.39	1.26
c. In pasteurized	23.7	17.0	39.2	131.2	6.28	1.63
d. In (c) after 24 hr.	29.4	17.3	32.5	131.7	6.32	1.08

TABLE 2

Influence of added calcium and phosphorus on the distribution of calcium, magnesium, and phosphorus in pure salt solutions and on the pH and Ca:P ratios

Treatments	Calcium	Magnesium	Phosphorus	pH	Ca:P ratios in sediment
	(mg./100 ml.)				
1. Total in sample	94.0	19.2	75.5	.....	.....
2. Total in sample with added P[a]	.....	.....	97.5	.....	.....
3. Total in sample with added Ca[a]	139.1	.....	.....	.....	.....
4. Soluble in untreated	33.8	14.0	44.7	6.67	1.48
5. Soluble in pasteurized	23.7	17.0	39.2	6.28	1.71
6. Soluble in pasteurized and cool-aged	29.4	17.3	32.5	6.32	1.16
7. Soluble in pasteurized after addition of phosphorus	17.2	15.0	45.3	6.65	1.14
8. Soluble in above after cool-aging	21.0	16.7	47.5	6.55	1.13
9. Soluble in pasteurized after addition of calcium	45.0	15.7	31.0	5.63	1.63
10. Soluble in above after cool-aging	50.5	11.9	25.6	5.25	1.37

[a] 22 mg. per 100 ml. phosphorus added as Na_2HPO_4, and 45 mg. per 100 ml. calcium as calcium acetate.

calcium, there was a simultaneous decrease in the pH, and vice versa. The variations in pH were the result of tricalcium phosphate precipitation. Magnesium phosphate precipitation must also be considered, because the solubility of this salt is different from that of tricalcium phosphate at higher temperatures.

On aging, there was only a slight increase in soluble magnesium in the untreated aliquot, whereas the pasteurized and aged portion showed even less increase in this fraction of magnesium. However, there was a decrease in the solubility of phosphorus from raw to pasteurized, which showed further decrease on cool-aging. This may indicate a reversion of tricalcium to dicalcium phosphate.

The behavior of citrate in milk is of interest. The prevention of clotting of milk with rennin by adding citrate is often regarded as resulting from the formation of un-ionized calcium citrate, which removes calcium ions from solution, these ions being considered essential for the clotting process. But Wiley (*10*) states that more citrate is present than is necessary for equivalence with calcium. Thus, if un-ionized tricalcium citrate were formed, there would

TABLE 3

Distribution of added calcium and phosphorus in the salt solution after various treatments

	Calcium	Phosphorus
	(mg. %)	
1. Added in 100 ml. of solution	45.0	22.0
2. Recovered in the supernatant from:		
(a) Pasteurized sample	21.3	6.1
Per cent of total	47.3	27.7
(b) Pasteurized and aged sample	21.1	15.0
Per cent of total	46.8	68.1

be no calcium ions in normal milk, and if calcium ions were necessary for coagulation, milk would not clot. Somehow, citric acid does not form much tricalcium citrate in milk; otherwise, based on its dissociation properties there would be practically no ionized calcium in milk. The true salt mixture also did not form much tricalcium citrate, and in this respect the behavior of the salt mixture and of the milk was alike. The effect of ionic concentration may be responsible in some way for the lack of formation of tricalcium citrate in salt mixtures. In milk, the situation is further complicated by the presence of other organic constituents. Another similarity in the nature of citrate in milk with salt mixture may be pointed out. Contrary to earlier reports *(2, 7)*, the citrate in milk is present in colloidal form to the extent of about 15% in rennet curd *(9)*, which also is likely to be the case in milk without rennet treatment. This also holds true for pure salt mixture.

The mole ratios of Ca:P in the insoluble phase of the salt solution were computed according to Verma and Sommer *(9)*. The variations in these ratios may indicate shifts of equilibria, rather than precise evidence of the exact salt combination. Thus, we see (Table 1) that the sediment in the untreated salt mixture had a Ca:P ratio of 1.46, which changed to 1.26 on cool-aging. Pasteurization increased the ratio to 1.63, which also decreased to 1.08 on cool-aging. These changes may be explained in terms of tricalcium phosphate precipitation on pasteurization and reversion to dicalcium phosphate on cool-aging. The pH of the mixture was reduced from 6.67 to 6.28 on pasteurization, with an increase to 6.32 on cool-aging. Qualitatively, this also was in harmony with the changes in tricalcium phosphate precipitation indicated from Ca:P ratios above.

It is seen (Table 2) that the pasteurized aliquot to which calcium was added had a Ca:P ratio of 1.63 as compared to 1.48 for the untreated control and 1.71 for pasteurized. In this table, the Ca:P ratios are calculated without the benefit of data oin citric acid, because these analyses were not included here. These ratios may not be compared to the figures in Table 1, except in a qualitative sense. The ratio in calcium-added milk decreased to 1.37 on cool-aging. The addition of calcium decreased the pH from 6.28 to 5.63 with further decrease during cool-aging. Phosphorus addition caused an increase in pH from 6.28 to 6.65, which decreased on cool-aging.

Increase of calcium and pasteurization both caused a decrease in the pH, but cool-aging did not increase the pH in the calcium-added aliquot within the period of 24 hr. It probably takes longer to aproach equilibrium. Phosphate-added aliquot showed lower Ca:P ratio and increased the pH. This is consistent with dicalcium phosphate formation. Cool-aging decreased Ca:P ratio in the sediment, but the decrease in the pH in this case was not much.

The distribution of the added salts into the supernatant and sediment as a result of the various treatments was computed and is shown (Table 3). The fate of the added calcium and phosphorus in the salt solution showed significant variation. Whereas the amount of calcium in supernatant from pasteurized and cool-aged treatments did not vary, the phosphorus showed significant differ-

ences—27.7 and 68.1%, respectively—for the two treatments. This also confirmed insoluble phosphate formation on pasteurization, which reverts to soluble phosphate on cool-aging.

It can also be seen (Table 1) that in 100 ml. of salt solution the amounts of different salts that remained undissolved upon centrifugation were: calcium, 60.2 mg., magnesium, 5.2 mg., phosphorus, 31.4 mg., and citric acid, 29.6 mg. If all the calcium in this were to form tricalcium phosphate, 60.2 mg. Ca would require 30.9 mg. P, leaving a remainder of only 0.5 mg. of P. Also, assuming that all the citric acid combined with Mg to form Mg-citrate, 29.6 mg. of citric acid would require 5.6 mg. of Mg, leaving a deficit of only 0.4 mg. of Mg. Thus, the salts remaining undissolved in the mixture made to simulate salts in milk are in substantial balance, on the basis of their occurrence as tribasic phosphates and/or citrates of calcium and/or magnesium. The influence of pasteurization and cool-aging of pasteurized aliquot, and of addition of calcium and phosphorus, also are understandable in terms of shifts of ionic equilibria. Thus, the deviations in the behavior of salts in milk from this pattern should be explained in terms of the influence of the other colloidal systems present in milk.

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USE OF FLUORESCENT MATERIALS FOR THE INDIRECT DETECTION OF ANTIBIOTICS IN MILK

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SUMMARY

The addition of a combination of fat-soluble fluorescein (Fluoral) and uranine, as a "marker," to penicillin preparations intended for intramammary infusion, was found to provide a rapid and satisfactory means for detecting the antibiotic in milk. The marker was detected visually in the milk for 48 hr. after treatment and with ultraviolet light for 96 hr. The marker was nontoxic to the treated animals and did not affect milk production. Statistical analysis of the data showed a close correlation between the excretion of marker and penicillin from the treated udders.

A rapid, sensitive, and reliable means for detecting antibiotics in milk is needed urgently by dairy manufacturers, dairy farmers, and public health officials. Although several sensitive and apparently reliable tests for antibiotics have been proposed recently, all of them are laboratory tests that require from 2½ to 8 hr. *(1, 3, 4)*. The time, laboratory equipment, and skill required for these tests reduce their usefulness, particularly to cheese-makers and farmers.

A rapid and practical test for antibiotics in milk would enable farmers to determine definitely how long to withhold the milk from antibiotic-treated cows; for manufacturers to avoid serious economic loss resulting from the injurious effects of antibiotics on bacterial starters used in making cheese and fermented milks, and for public health officials to make routine tests of large numbers of milk samples.

The incidence and concentrations of antibiotics in market milk have been reported recently *(2, 5, 6)*, and cheese-makers have frequently encountered complete failure of bacterial starters, owing to the presence of antibiotics in the milk.

In 1950, the Agricultural Research Service encountered sporadic appearances of antibiotics in the milk being used for making experimental cheese. At that time, the feasibility of incorporating a dye in all antibiotic preparations to be used for intramammary infusion was investigated. Such a dye would serve as a visual index of antibiotic contamination in milk. The ideal dye for this purpose would have to remain in the milk as long as the antibiotic, have no effect on the antibiotic activity, and be nontoxic and nonirritating to the cows' udders. Although some of the dyes tested in the early studies were readily detectable visually for 48 hr. after their infusion into udders, they failed to remain in the milk as long as did penicillin.

Received for publication October 3, 1957.

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Recently, these studies have been resumed, using fluorescent materials and additional dyes as antibiotic markers. Although several phases of the work remain to be completed, preliminary experiments indicate that it is feasible to add such markers to antibiotics intended for veterinary use. This report briefly presents the pertinent findings.

EXPERIMENTAL PROCEDURE

Various chemicals were screened, to find an agent that could be used as a tracer, or marker. Selection was based primarily upon fluorescence, color, and odor. The list of chemicals tested included certified food colors, cosmetic dyes, food-flavoring compounds such as vanillin, cinnamaldehyde, and methyl anthranilate, odoriferous materials such as furfuryl mercaptan and gamma undecalactone, and various fluorescing agents such as the fluoresceins, chlorophylls, and hydroxycoumarins. In the preliminary study, each material was tested for ease and limits of detection in whole milk. Milks containing fluorescent materials were diluted serially and measured for fluorescence with a 2-amp. long-wave ultraviolet (3660Å) lamp. Effects of the chemical upon the antibiotic activity of penicillin were determined by penicillin assays with *Streptococcus thermophilus*. Materials that appeared to have value as tracers were tested further, for their possible effects on antibiotic activity during storage. In most instances, mixtures of the chemical and penicillin in mineral oil and also in a water-in-oil preparation (Penicle),[2, 3] were stored at room temperature. The limits of detection for tracer agents were established visually by a number of individuals, both under normal and under ultraviolet light. Agents selected on the basis of the in vitro tests were subjected to further tests, by injection into udders, either alone or in combination with penicillin.

Three separate trials were conducted to evaluate the marker materials in the in vivo studies. Some markers were subjected to additional trials if further testing seemed advisable. Each trial was designed as a balanced incomplete block experiment, with four marker treatments assigned at random to the four quarters of the udder of each of six cows representing a wide range in milk production. A common source of oil-penicillin preparation was used in preparing the emulsions of tracer materials. In every instance, one 15-ml. dose, containing known amounts of marker, oil, and penicillin, was administered. The dose of penicillin ranged from 100,000 to 300,000 units, and the dose of tracer from 250 to 400 mg. Quarter-milkers were used to collect samples of milk from the individual quarters for at least 96 hr. after infusion. Quarter-milkings prior to treatment were used as controls for pH measurements and leucocyte counts. Close veterinary scrutiny and complete milk production records were maintained. Milk samples from the treated quarters were tested for leucocytes, pH, penicillin content, and for the presence of marker. Further, milks containing marker

[2] Pitman and Moore.

[3] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

were diluted with normal herd milk to determine the extent of dilution without loss of marker detection.

Trial I. 4-Methyl umbelliferone, methyl anthranilate, esculin, and uranine (sodium fluorescein) were used as marker materials. Each test dose contained 250 mg. of marker and 100,000 units of penicillin G in 15 ml. of an oil base containing equal parts of light mineral oil and petrolatum.

Trial II. Uranine, oil-soluble fluorescein (Fluoral 7 Ga.)[3, 4] a combination of uranine and oil-soluble fluorescein, and spirit-soluble fluorescein were compared as marker materials. Each dose contained 250 mg. of marker and 100,000 units of penicillin in an oil base. The oil base in this trial was Penicle.

Trial III. Esculin, a combination of esculin and Amaranth Red No. 2, oil-soluble fluorescein, and oil-soluble chlorophyll were tested. Each test dose contained 400 mg. of marker and 300,000 units of penicillin G in 15 ml. of Penicle.

Data accumulated from these trials were statistically analyzed to evaluate the marker materials, as well as to determine the effect of the level of milk production on the rate of excretion of the antibiotic and marker from the udder.

RESULTS

The dyes tested to date do not seem to have as much potential as tracer materials as do the fluorescing agents. For the most part, the odorous or flavored materials were unsatisfactory as tracer agents. The limits of identification for these materials varied tremendously from one individual to another, and to a lesser degree with milk quality. In this report, only the marker materials that have been tested by udder infusion will be considered.

Limiting concentrations of marker material at which color of fluorescence was apparent in milk are shown (Table 1). Laboratory attempts to isolate tracer materials from the milks were successful with a few agents, but such procedures were not considered to be practical for routine identification. The fluorescein derivatives could be detected in milk both by color and by fluorescence in rather high dilution. As may be noted, esculin and 4-methyl umbelliferone were detected in milk only by the use of ultraviolet light. Methyl anthranilate was detected by ultraviolet light and also by its grape-like odor and taste.

None of the markers caused a degradation in the antibiotic activity of penicillin stored in mineral oil at room temperature over a period of 4 mo. In the

[4] General Dyestuff Company.

TABLE 1

Limiting concentration of marker detected in whole milk

Marker	Ordinary light	Ultraviolet light
	(p.p.m)	
Esculin	Not detectable	1
Uranine	200	10
Oil-soluble fluorescein	200	10
Spirit-soluble fluorescein	100	10
4-Methyl umbelliferone	Not detectable	1
Methyl anthranilate	Not detectable	10
Amaranth Red No. 2	100	Nonfluorescent
Oil-soluble chlorophyll	200	200

Penicle preparation stored at room temperature, Amaranth Red caused a marked reduction in penicillin activity. The limits of identification for the markers in milk were not affected by storage with penicillin in any of the oil preparations.

In the in vivo studies, it became evident that some of the materials were unacceptable as tracer agents, whereas others showed considerable promise. Some agents appeared to be slightly toxic and others were absorbed from the udder. Marker agents were evaluated after a statistical analysis of the data from the penicillin and marker determinations on the treated milks from individual cows. The values listed (Table 2) are the averages for the six cows used to test each marker. These data show the number of milkings after treatment during which penicillin was being shed in the milk at different levels, and the number of milkings during which the markers could be detected at various dilutions. In every instance, penicillin was detected at the 72-hr. period and, in many instances, at 96 hr. or longer. This emphasizes the need for a test to detect antibiotics in milks as long as they are excreted, rather than depending on a fixed period of 72 hr. for the milk to be withheld.

Trial I. The persistency of the marker materials in the udder did not appear statistically different. However, esculin and uranine were definitely superior with respect to detectability in the treated milks. The veterinary report indicated clearly that 4-methyl umbelliferone and methyl anthranilate were unsuitable as marker agents. 4-Methyl umbelliferone caused a slight but definite irritation in all the test quarters, whereas methyl anthranilate, although nontoxic, partially was absorbed in the udder and appeared in the urine of the cows. No rise in leucocyte count or pH of the milk was noted either with esculin or with uranine.

Trial II. In this trial, the marker persistency at the zero and 1 : 4 dilutions showed a highly significant difference between markers. Oil-soluble fluorescein had the greatest persistency and was readily detected in the herd milk. The leucocyte count and pH of treated milks remained constant.

Trial III. In Trial III, marker persistency at the zero and 1:4 dilutions showed highly significant differences between markers. However, the differences between esculin, a combination of esculin and Amaranth Red, and oil-soluble fluorescein were not significant. Chlorophyll was definitely inferior to the three other test materials. (Results of recent tests with increased doses of chlorophyll suggest that it has much greater value as a marker than is indicated in Table 2.)

The combination of uranine and oil-soluble fluorescein was very effective as a marker, in that it definitely colored the milk for at least 48 hr. after infusion, and was detectable by ultraviolet light for 96 hr. after infusion.

The retention of penicillin and marker in the quarter was influenced greatly by the quantity of milk being produced. Low producers retained penicillin and marker in the quarter as much as 24 to 48 hr. longer, and at higher levels, than did the higher producers.

Increasing the penicillin dose from 100,000 to 300,000 units had little effect upon the number of milkings in which penicillin persisted. However, a greater proportion of penicillin appeared in the earlier milkings with the larger dose.

TABLE 2

Persistence of penicillin and markers in milk

Trial	Marker	Marker dose	Penicillin dose	Av. milk yield per quarter	Penicillin detected at levels of: 0.1 (units/ml)	0.2 (units/ml)	1.0 (units/ml)	Marker detected at dilutions of: 0	1–4	1–9
		(mg.)	*(units)*	*(lb.)*	*(Av. No. milkings)*					
I	1. 4-Methyl umbelliferone	250	100,000	.....	6.25	5.34	3.22	2.05	0.55	0.02
	2. Methylanthranilate	250	100,000	.....	7.79	6.71	4.18	1.31	0.19	0
	3. Esculin	250	100,000	.....	7.01	6.06	3.86	9.10	5.88	3.77
	4. Uranine	250	100,000	.....	7.17	5.88	3.22	10.24	6.03	3.35
II	1. Uranine	250	100,000	3.01	8.24	7.24	4.90	2.73	1.58	0.92
	2. Oil-soluble fluorescein	250	100,000	3.41	7.34	6.50	4.37	8.70	5.36	3.16
	3. Combination uranine and oil-soluble fluorescein	125 (each)	100,000	4.00	8.00	7.06	4.73	5.51	3.73	2.55
	4. Spirit-soluble fluorescein	250	100,000	3.92	7.44	6.54	4.45	4.57	3.15	2.20
III	1. Esculin	400	300,000	3.88	7.15	6.45	4.81	7.69	5.96	4.81
	2. Combination, Amaranth Red and esculin	400 (each)	300,000	3.71	7.24	6.50	4.77	7.54	5.88	4.78
	3. Oil-soluble fluorescein	400	300,000	3.71	7.18	6.48	4.84	7.96	5.47	3.82
	4. Oil-soluble chlorophyll	400	300,000	3.78	8.03	7.21	5.30	2.94	0	0

Increasing the marker doses had a similar effect, especially with the water-soluble agents. The oil-soluble materials appeared to remain in the udder slightly longer and at a higher concentration level than did most of the water-soluble agents.

Table 3 shows the correlation coefficients between penicillin and marker persistency. The correlation was obtained for the over-all milkings as well as for the within-milkings. It is of interest to note that a combination of uranine and oil-soluble fluorescein was more closely associated with penicillin persistency than any other marker, or combination of markers.

In Trial II, an increase of 1 lb. of milk yield decreased penicillin persistency (at a level of 0.10 penicillin unit) by 0.28 milking. In Trial III, an increase of 1 lb. of milk yield decreased penicillin persistency by 0.115 milking. The fact that milk yield had less effect on penicillin persistency in Trial III might be attributed to the fact that dosage of penicillin administered in Trial III was much greater than in Trial II.

In Trial II, the statistical analysis of data showed that an increase in 1 lb. of milk yield decreased marker persistency at the 1:4 dilution level by 0.31 milking. In Trial III, this decrease was 0.145 milking. Again, the dosage of the marker was greater in Trial III than in Trial II. It is of interest to note the strong parallel in the influence of milk yield on the persistence both of penicillin and of marker.

TABLE 3

Correlation coefficients between penicillin and marker persistency

Trial	Marker	Milkings	
		Total	Within
I	4-Methyl umbelliferone	0.67	0.07
	Methyl anthranilate	0.22	−0.10
	Esculin	0.91	0.84
	Uranine	0.90	0.85
II	Uranine	0.75	0.45
	Oil-soluble fluorescein	0.81	0.61
	Combination of oil-soluble fluorescein and uranine	0.94	0.82[a]
	Spirit-soluble fluorescein	0.85	0.22
III	Esculin	0.88	0.26
	Combination of Amaranth Red and esculin	0.86	0.20
	Oil-soluble fluorescein	0.88	0.34
	Oil-soluble chlorophyll	0.31	−0.19

[a] Perhaps this indicates that a combination of 250 mg. of oil-soluble fluorescein and 125 mg. of uranine is most closely associated with penicillin persistency.

DISCUSSION

Hydroxycoumarin derivatives such as esculin, umbelliferone, and 4-methyl umbelliferone are known for their fluorescence in ultraviolet light. Small quantities were easily detected in milk by their fluorescence. Esculin has been reported to be detectable in water at a concentration of 0.1 p.p.m., and we found it to be strongly fluorescent in milk.

The optimal pH for fluorescence was considered for all materials. The fluorescence of 4-methyl unbelliferone in milk was increased tenfold by adjusting the

milk pH to 9.0. The brilliant blue fluorescence of esculin was more difficult to detect in old milk, as there was a tendency for the milk, as it aged, to change from a greenish to a blue fluorescence. Methyl anthranilate was selected as a possible tracer agent, because it can be added to foods, has a strong grape-like aroma and flavor and, in addition, is fluorescent in ultraviolet light. Although methyl anthranilate and 4-methyl umbelliferone could be extracted in *n*-butyl alcohol, they proved to be unsatisfactory in other respects in the in vivo studies.

Plant chlorophyll is known to produce a fiery-red fluorescence and, as it has been reported to have some medicinal properties, it seemed an excellent material to include in the study. Of the chlorophyll preparations tested, the oil-soluble form showed more potential as a marker. Small quantities in milk could be detected by extraction in *n*-butyl alcohol.

Fluorescein derivatives, as might be expected, gave a bright-green fluorescence and color to the milk. Some difficulty may be encountered in differentiating the yellow fluorescence of uranine from that of normal milk in concentrations of 1 p.p.m. or less. However, adjustment of the milk to pH 7.5 to 8.0 aids in the detection of uranine by diminishing the normal fluorescence of milk. Oil-soluble fluorescein appeared in the treated milks attached to the fat globule. The floating fluorescent globules were easily detected in the fluid milk by ultraviolet light.

In our studies, no evidence of toxicity was noted when esculin, chlorophyll, uranine, and oil-soluble fluorescein were injected into the udders of cows. A lack of toxic effects of these agents when ingested by animals has not been determined in this study. The cost of marker material is an important factor in considering it for commercial use. The amount of oil-soluble fluorescein and uranine added to the veterinary antibiotic preparation should not increase the cost more than one-half cent per dose.

ACKNOWLEDGMENTS

The authors wish to thank the Dairy Husbandry Department, University of Wisconsin, for the loan of the quarter-milkers used in this study.

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USE OF ANNATTO FOR COLORING BUTTER[1]

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SUMMARY

A comparison was made of the effect of annatto and coal-tar color on the chemical quality of stored butter. Commercial conditions were duplicated by selecting two levels of cream quality, two levels of copper, and two storage treatments.

Flavor was evaluated by official graders and by a panel of 15 persons. The surface color was determined with a photoelectric reflection meter and also with Dyeco color standards. Peroxide values were determined by the ferric thiocyanate method; aldehyde values by the rosaniline method; acid degrees by titration; copper content by the carbamate method, and vitamin A by the Carr-Price method.

Official graders and a taste panel agreed on a difference in flavor between high- and low-copper samples. The flavor of the high-copper samples decreased on storage. No flavor difference was attributable to the type of color. Peroxide and aldehyde values increased on storage in the samples with high copper content, but the type of coloring agent was not a factor. Acid-degree values increased slightly on storage, but were not affected by the type of color or by the level of copper. Carotene accounted for 10% of the vitamin A potency of the butter; it was not affected by storage for 6 mo. at −5° F., but decreased following additional storage for 2 wk. at 60° F. The preformed vitamin A content of the butter was not affected by the type of color, copper level, or storage.

Annatto color has been used in butter since the early days of the butter industry. During the last few decades, in the United States and Canada, it has been largely replaced by coal-tar dyes, although there are countries where annatto color has remained the main butter color.

For several years the color of American and Canadian butter has been standardized almost exclusively with the coal-tar dyes Yellow AB and Yellow OB. The alleged toxic properties of these dyes and the need for other coloring agents for butter have been discussed in a previous paper *(13)*. The pharmacological aspects of annatto color have only recently received consideration. Engelbreth-Holm and Iversen *(4)* indicated that the vegetable annatto butter color can not be considered cancrogenic to rats and mice. Notwithstanding the extensive use of annatto in butter, the chemical effect of this color on the quality of stored butter has never been studied comprehensively. This was the object of the investigation described in this paper.

EXPERIMENTAL PROCEDURE

Manufacturing. The factorial experiment involved two types of color, two levels of cream quality, two levels of copper, and three storage treatments. The butter was made in April, 1956, from split vats of Special Grade and First Grade cream *(1)*. The Special Grade cream was obtained from the Experimental Farm Dairy. Its initial acidity was uniformly 0.14%. The First Grade cream,

Received for publication July 2, 1957.

[1] Contribution No. 368.

obtained from a local milk plant, had an initial acidity of 0.20 ± 0.02%, and a reasonably clean flavor. The fat content of the cream was 32% in each vat. The acidity was standardized to 0.10% acidity with soda neutralizers. Lots of cream from two sources were pasteurized separately in a vat of 3,000-lb. capacity at 170° F. for 10 min., then cooled to and maintained at 45° F. overnight. Each vat of cream was split into four lots, the respective lots being treated with *(a)* annatto color; *(b)* annatto color plus 3 p.p.m. copper; *(c)* coal-tar color, and *(d)* coal-tar color plus 3 p.p.m. copper.

Coal-tar color (Hansen) was added at the rate of 1 oz. per 100 lb. of fat in 12 lots of cream. To approach the color of the butter colored with coal-tar, annatto color (Hansen) was added to six comparable lots of cream at the rate of 3 oz. per 100 lb. of fat, and to six other lots at 4 oz. per 100 lb. of fat. It was assumed that this difference in the level of annatto color had no effect on the butter other than a difference in color intensity. All annatto-colored samples were treated as one group, for the analysis of variance of all values except color values.

Copper was added as a 0.5% solution of copper sulfate, at the rate of 3 p.p.m. of the cream, in 12 comparable lots involving the two types of color and the two levels of cream quality. This addition raised the copper content of the resulting butter from 0.04 to 0.70 p.p.m.

Annatto color, coal-tar color, and copper were added to the cream in the churn. The cream was churned at 53° F. The butter was salted at the 2.0% level.

Samples from each churning were examined initially. Two 7-lb. blocks of butter from each churning were wrapped in parchment paper, boxed, and stored. One of the two stored samples from each churning was examined after 6 mo. at −5° F., and the other, after 6 mo. at −5° F. plus 2 wk. at 60° F.

Methods of analysis. Three government graders scored the samples for flavor and evaluated the color by comparison with Dyeco color standards.[2] Each set of four comparable butters was ranked for flavor from one to four, in decreasing order of preference, by a taste panel of 15 persons.

Butter oil was prepared by melting and filtering the butter at 122° F. just before analysis for peroxide, aldehyde, acid-degree, and color values. Peroxide values were determined by the method of Lips *et al. (10)*. Schibsted's method *(14)*, as modified by Mummery *(12)* and Dolby *(2)*, was used for aldehyde values. The latter were expressed in terms of density value × 100, at 510 mμ, using a 1-cm. cell and a 5% fat solution. The fat acid degrees were expressed in ml. of 0.050 *N* sodium ethylate per 5 g. of fat. They were determined by titrating 5 g. of the butter oil in 50 ml. of 1 : 1 benzene-ethanol to the phenolphthalein end-point with 0.0040 *N* sodium ethylate. The colors of the butter oils were objectively determined by measuring the optical density at 450 mμ of a 20% solution of the oil in benzin. The color values were reported as γ β-carotene per g. of fat; the extinction coefficient was $\Sigma_1^{1\%/ml}_{cm.} = 0.270$.

The surface color of the butter was determined objectively with the use of a Photovolt photoelectric reflection meter, Model 610. The tristimulus values and

[2] Dye and Chemical Co. of Canada Ltd., Kingston, Ontario, Canada.

the Hunter *a*, *b*, and *L* values were calculated according to equations given by Judd *(7)*. The copper contents of the butter were determined by the method described by McDowell *(11)*. The method of Koehn and Sherman *(9)*, as described by Jenness and Palmer *(6)*, was used to determine the vitamin A potency of the butters.

RESULTS AND DISCUSSION

Color. The average yellow color produced by 3 oz. of annatto color per 100 lb. of fat was equivalent to 6.05 γ of β-carotene per g. of fat, and to a *b* value of 30.08. That of their comparable churnings colored at the rate of 1 oz. of coal-tar color per 100 lb. of fat was equivalent to 7.56 γ of β-carotene per g. of fat, and to a *b* value of 32.30. That these color differences were significant at P = 0.01 is shown in Table 1. The churnings comparing 4 oz. of annatto color and 1 oz. of coal-tar color per 100 lb. of fat were not significantly different in yellow color. It was estimated that 3.7 oz. of annatto color had approximately the coloring power of 1 oz. of coal-tar color.

TABLE 1

Analysis of variance of color values

Source of variation	Degrees of freedom	Mean square			
		As β-carotene	*a* value	*b* value	*L* value
Annatto *(3 oz/100 lb. fat)*					
Color	1	20.44[a]	18.45	44.74[a]	0.24
Copper	1	0.08	0.02	12.82	0.16
Color × copper	1	0.20	0.00	0.14	0.55
Storage	2	2.90	2.35	7.74	0.98
Storage × color	2	0.16	0.02	0.54	0.06
Storage × copper	2	0.22	2.43	4.70	0.05
Error	26	0.99	6.28	3.11	1.08
Annatto *(4 oz/100 lb. fat)*					
Color	1	0.03	22.97[b]	0.14	13.12[a]
Copper	1	1.21[b]	1.35	5.53[b]	0.00
Color × copper	1	1.22[b]	3.21	0.48	0.05
Storage	2	1.13[b]	0.35	3.35	0.02
Storage × color	2	0.21	0.42	0.38	0.50
Storage × copper	2	0.36	2.54	2.05	0.65
Error	26	0.26	3.88	1.05	0.57

[a] Significant at P = 0.01.
[b] Significant at P = 0.05.

The mean *a* values for the comparable churnings colored at the rate of 3 oz. of annatto color, and at the rate of 1 oz. of coal-tar color per 100 lb. of fat were, respectively, −1.71 and −3.14. Those for the comparable churnings colored with 4 oz. of annatto color and with 1 oz. of coal-tar color per 100 lb. of fat were, respectively, −1.46 and −3.06. Higher *a* values indicate more redness, but Table 1 shows that the differences in *a* values were significant only in the latter comparison.

The degree of lightness was similar for the comparable butters colored with 3 oz. of annatto color and with 1 oz. of coal-tar color per 100 lb. of fat. The mean *L* values were 87.99 and 88.99, respectively, for the comparable samples colored with 4 oz. of annatto color and with 1 oz. of coal-tar color per 100 lb. of fat.

Table 1 shows that this difference was significant at P = 0.01.

The butter colored with 1 oz. of coal-tar color per 100 lb. of fat differed by 2.0 and 2.7 NBS units, respectively, from that colored with 3 and 4 oz. of annatto color per 100 lb. of fat.

Copper and storage effected a slight decrease in the degree of yellowness in the group of samples colored with 4 oz. of annatto color per 100 lb. of fat.

Flavor. A taste panel of 15 persons ranked for flavor the comparable butters colored either with annatto or with coal-tar, at two levels of copper. A difference between some of these treatments was indicated by the coefficients of concordance *(8)* significant at P = 0.01 (shown in Table 2). This was corroborated by the analysis of variance of the normal deviates *(5)*. Using the multiple range test of Duncan *(3)*, it was established that this difference concerned only the copper treatment, and that the panel members favored the low-copper samples, except for the coal-tar versus annatto plus copper at initial ranking. In no instance did the taste panel find the flavor to be influenced by the type of color used. That the type of color did not affect the flavor of the butter as scored by official graders is shown (Table 2).

TABLE 2

Effect of type of color on flavor of stored butter

Treatment	Storage		
	Initial	6 mo., −5° F.	6 mo., −5° F. plus 2 wk., 60° F.
Flavor score:			
Chi-square of flavor score	5.07	5.33	2.09
Degrees of freedom	2	3	3
Flavor ranking:			
Av. No. judgments [a]	15	13	13
		Av. sum of rankings[a]	
Annatto	32.2	30.2	21.5
Coal-tar	37.0	27.3	24.7
Annatto plus copper	38.3	36.0	40.3
Coal-tar plus copper	42.5	36.5	43.5
Concordance coefficient	14.94 [b]	5.93 [b]	53.91 [b]

[a] Average of six replicates.
[b] Significant at P = 0.01.

Chemical variables. Table 3 shows that the type of color had no effect on peroxide and aldehyde values. In the samples low in copper content (0.04 p.p.m.), the peroxide and the aldehyde values did not change on storage. High copper, however, increased both values on storage. After 6 mo. at −5° F. plus 2 wk. at 60° F., the peroxide value had increased from 0.39 to 3.16 and the aldehyde value from 3.25 to 60.93. Similar observations were made in a previous study, which compared β-carotene with coal-tar color *(13)*.

The acid-degree values averaged 0.82 in the fat from Special Grade cream and 1.21 in fat from First Grade cream. Storage increased the acid-degree value from 0.95 to 1.07. Table 3 shows that these two differences were significant at P = 0.01. The type of color and the level of copper had no effect on the acid degree value.

Vitamin A potency. In these butters manufactured from winter cream, caro-

TABLE 3

Analysis of variance of peroxide, aldehyde, acid-degree values, and of vitamin A potency

Source of variation	Degrees of freedom	Mean square			
		Peroxide value	Aldehyde value	Acid-degree value	Vitamin A potency
Color	1	0.1398	144.87	0.0018	1.2366
Cream	1	0.0210	218.37	2.7067[a]	0.2901
Copper	1	38.4695[a]	11,637.02[a]	0.0011	1.6720
Color × cream	1	0.0002	20.44	0.0012	0.4711
Color × copper	1	0.3724	129.26	0.0001	0.2667
Cream × copper	1	0.0528	191.85	0.0006	0.0819
Storage	2	11.3234[a]	5,413.94[a]	0.0962[b]	3.9104[a]
Storage × color	2	0.0515	36.01	0.0025	0.4763
Storage × cream	2	0.0754	126.33	0.0332	0.1522
Storage × copper	2	11.8140[a]	4,788.99[a]	0.0001	0.0882
Error	57	0.9634	54.90	0.0213	0.5623

[a] Significant at P = 0.01.
[b] Significant at P = 0.05.

tene accounted for 10% of the total vitamin A potency. Storage for 6 mo. at −5° F. did not affect the carotene content of the butter. Exposing the butter for an additional 2 wk. at 60° F., however, effected an 18% decrease, significant at P = 0.01, in the carotene content.

The preformed vitamin A content was not affected by the type of color, copper level, or storage.

The total vitamin A potency of the butter averaged 10,500 I.U. per lb. before storage. That storage effected a slight decrease, significant at P = 0.01, in the total vitamin A potency of the butter, is illustrated in Table 3.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Messrs. D. C. Beckett, D. M. Beattie, O. H. J. White, and J. G. MacMillan in the processing and grading of the butter.

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ESTROGEN CONTENT OF COLOSTRUM AND MILK OF DAIRY CATTLE [1, 2]

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SUMMARY

A study of the permeability of the secreting mammary gland epithelial cell to circulating blood estrogens is reported. Milks were dried and fed to ovariectomized mice for ten days. The increase in uterine weight of these mice was compared to controls and to those fed graded amounts of diethylstilbestrol added to dried whole milk, to estimate the estrogen in milk. Milks from 11 nonpregnant cows, producing from 26.9 to 66.8 lb. per day, were assayed. The average uterine weight of the group was 10.48 mg., and uterine weight/% of body weight was 0.05. The milk of nonpregnant cows either is free of estrogen or it contains amounts which can not be detected by the assay method. Milks from 32 cows, pregnant from 27 to 268 days, were assayed. The milk of ten cows, pregnant from 27 to 97 days, averaged mouse uterine weights of 11.35 mg., with an average uterine weight/% body weight of 0.06. Milk of 11 cows, from 104 to 193 days pregnant, averaged uterine weights of 13.71 mg., with an average uterine weight/% body weight of 0.07. Milk from 11 cows, from 200 to 268 days pregnant, averaged uterine weights of 16.45 mg., with an average uterine weight/% body weight of 0.09. These data indicate that the normal lactating cells are only slightly permeable to the normal circulating estrogen of the cow's blood, even in late pregnancy, when estrogen secretion is high and secretion of milk is low. Commercial market milk samples were obtained monthly for 18 mo. The average uterine weight of mice was 11.32 mg. and uterine weight/% body weight was 0.06. Samples of colostrum from six normal cows and from three cows experimentally induced to lactate showed low uterine weight increases. These observations are significant, because the secretion of estrogen in late pregnancy is at a maximum and the rate of colostrum (milk) secretion is minimal. Seven nonpregnant cows, producing from 5.4 to 26.8 lb. per day, were fed diethylstilbestrol at from 10 to 20 mg. per day per 1,000 lb. live weight, for 2 wk. or more. Nine samples from cows fed 10 mg/day showed average uterine weight of 12.07 mg. and an average uterine weight/% body weight of 0.07. These data indicate that the secreting mammary gland epithelial cell either is nonpermeable or is only slightly permeable, to circulating natural estrogenic hormones or to synthetic estrogens (diethylstilbestrol) when fed at levels of from 10 to 20 mg/day/1,000 lb. body weight.

The normal pathways of excretion of estrogen in cattle, via urine *(13)* and feces *(6)*, have received study during the past 25 yr. Recently, El-Attar and Turner *(3)* studied the combined estrogen excretion in the urine and feces simultaneously, during various stages of pregnancy in cattle.

The problem of the permeability of the secreting mammary gland epithelium to blood estrogens, i.e., the presence of estrogen in the milk of dairy cattle, has received relatively little study. There are three phases of the problem to consider, the estrogen in colostrum which is secreted, in part, in late pregnancy and stored in the udder until after parturition (the period of bagging up); the estrogen in normal milk during recurring estrous cycles, and the estrogen in the milk during pregnancy. This latter period is of greatest importance, because

Received for publication November 1, 1957.

[1] Contribution from the Missouri Agr. Expt. Sta., Journal Series No. 1809. Approved by the Director.

[2] This investigation was supported in part by a grant from the American Cancer Society.

[3] With the technical assistance of Mary E. Powell.

it is a time when the milk is used for human consumption, whereas the colostrum is usually fed to the calf.

Dairy cattle usually are bred between the 60th and 120th days of lactation, so that they will calve at yearly intervals. The estrogen secretion of the pregnant dairy cow is at first low, but increases slowly during the first 160 to 170 days. A rapid increase in estrogen secretion then occurs until parturition *(3)*. During the latter part of pregnancy, when the estrogen secretion is relatively high, the secretion of milk is declining. If the mammary gland is permeable to estrogen, one should expect considerable concentration in such milk or in the colostrum.

The present paper will report the results of an extensive study concerned with the assay of colostrum, milk from nonpregnant cows, and milk from cows during various stages of pregnancy, for the presence of estrogen. These data will be compared with assays of milk from cows fed low levels of diethylstilbestrol.

Courrier *(1)* observed that if daily doses of from 80 to 100 rat units of estrogen were given to lactating guinea pigs, after three or four days the nurslings showed opening of the vagina and mammary gland development. Lacassagne and Nyka *(4)* reported that human colostrum in amounts of 1.5 ml., injected subcutaneously, caused vaginal cornification in spayed mice. Walker and Stanley *(17)* injected 0.2 and 1.0 mg. of diethylstilbestrol dipropionate daily in castrate and intact lactating rats. Premature opening of the vagina of the nurslings occurred in most of the treated litters, indicating the presence of estrogen in the mothers' milk. Recently, Pope and Roy *(11)*, using chemical extraction procedures, reported that the estrogenic activity of bovine colostrum was of the same order as that of human colostrum, and of bovine and human pregnancy blood, but was much lower than that in bile and urine during pregnancy. The estrogens occurred mainly as conjugates in the aqueous phase of colostrum and were partly ketonic. The conjugated estrogens of separated colostrum from one cow were equivalent to 5.2 μg. estradiol-17β per liter. Munch *(9)* reported the presence of estrogen in the milk of Spotted Mountain cattle. Variations were observed in the estrogen concentration during estrous cycle and pregnancy.

In England, the effects of implants of diethylstilbestrol and hexestrol upon the initiation of lactation have been studied at Cambridge University and at the National Institute for Research in Dairying. Samples of milk from heifers there were assayed for the presence of estrogen by Lawson *et al. (5)*. It was reported that the amount of estrogen present was too small for assay by direct feeding to rats. Milk samples were then extracted and assayed by subcutaneous injection or intravaginal application in ovariectomized rats. Milk from cows implanted with diethylstilbestrol showed amounts varying from 0.4 to 1.0 μg. per pint; whereas, with hexestrol the estrogen varied from 0.01 to 0.5 μg. per pint.

Chas. Pfizer and Company *(10)* assayed the milk of cows fed 10 mg. of diethylstilbestrol daily. The milk was spray-dried. Ovariectomized mice received 3 g. of the dried milk daily for ten days. It will be noted that the uterine weight of the mice fed milk from the diethylstilbestrol-fed cows was lower than the control cows' milk.

TABLE 1
Assay of milk from normal cows and cows fed diethylstilbestrol
(Pfizer, 1955)

Source of milk	Mice	Body weight	Uterine weight	Uterine weight / Body weight
	(No.)	*(g.)*	*(mg.)*	*(%)*
Control cows	10	25.40	19.16	0.08
Stilbestrol-fed cows	10	26.60	16.38	0.06

Voelker and Becker *(16)* implanted diethylstilbestrol pellets containing 15, 30, 240, and 1,500 mg. into lactating cows. The milk was homogenized and fed to immature normal mice as the liquid part of their diet for seven days. Uterine weight increase was used as an index of the presence of estrogen. At low levels the uterine response was variable or absent; whereas, significant amounts of estrogenic activity were observed in the milk from cows implanted with the higher levels of diethylstilbestrol.

METHOD OF BIOASSAY OF MILK

The method of the bioassay of milk was a modification of that employed in the bioassay of residual estrogens in the tissues of steers *(12)*. The mice used in the assay were ovariectomized and the uterus allowed to involute for a period of ten days. During this period, the animals were fed laboratory mouse feed. The problem of the best mode of feeding the milk was studied. Liquid milk from three cows was fed to groups of ten mice, being supplied in a water bottle and cup. The body growth of these mice was poor and several failed to survive the ten-day assay period. It was concluded that the mice did not consume sufficient liquid milk to meet their nutritional requirements.

It was then decided, in subsequent work, to dry the milk under infrared heat lamps. By frequent stirring, a crumbly, dried product was obtained without scorching. The samples were then stored in the deep freezer until assayed. To a few groups, 90% dried milk and 10% ground mouse feed were fed. In all other assays, the dried milk was fed ad libitum as the exclusive source of feed to groups of ten mice for a period of ten days. The mice were then sacrificed, body weight determined, and the uterus dissected clear of fat and connective tissue and weighed immediately upon a Roller-Smith balance. Care was taken in dissection to cut the uterus cleanly at the vaginal end of the cervix.

Sensitivity of the Assay Method. In a previous report *(12)*, the effect of the addition of graded amounts of diethylstilbestrol to beef rib-eye tissue upon the uterine weight response of mice was determined. In the present report, a similar study was made, in which graded amounts of diethylstilbestrol were added to dried milk. The dried milk consisted of commercial dried nonfat milk, 450 g., to which was added 150 g. melted butter, giving a total of 600 g. of whole dried milk. The diethylstilbestrol was dissolved in an olive-oil carrier at the level of 0.9 μg/ml. Three ml. of the oil was mixed with 600 g. of dried milk. Each gram of dried milk contained 0.045 μg. of diethylstilbestrol. Each succeeding milk sample was increased by 3 ml. of oil containing the estrogen (Table 2). One control group of

TABLE 2

Effect of graded doses of diethylstilbestrol added to dried whole milk (25% fat)
(Ovariectomized young mature mice)

Amount of diethylstilbestrol	Mice	Mean body weight	Mean uterine weight		Uterine weight / Body weight	
(μg/g milk)	*(No.)*	*(g.)*	*(mg.)*		*(%)*	
Control (mouse feed)	7	26.9	11.8		0.04	
Control (dried milk)	10	22.4	12.9	11.2	0.06	0.05
Control (dried milk)	10	21.4	9.5		0.05	
0.045	10	19.3	10.0		0.05	
0.090	10	20.1	10.8		0.06	
0.135	10	15.2	15.3		0.11	
0.180	8	18.1	26.2		0.15	
0.225	10	16.3	27.3		0.17	
0.270	10	18.5	30.6		0.17	
0.315	10	20.1	39.3		0.20	
0.360	10	21.4	50.5		0.25	
0.405	10	21.5	57.2		0.27	

mice was fed commercial mouse feed. The results on control groups fed the whole dried milk are presented. The first of these two assays was conducted several months earlier than the second, which was conducted simultaneously with the assay of the graded amounts of estrogen. It will be noted that as the amount of diethylstilbestrol added to the dried milk increased, the mean uterine weight and the percentage of body weight gradually increased. These data may be referred to in estimating the diethylstilbestrol-equivalent in the milk of dairy cattle.

It will be noted that the addition of 0.045 and 0.09 μg/g dried milk did not stimulate the average uterine weight of the mice above the range of control values, although the assay of the control sample, run simultaneously with the graded amounts of estrogen, was lower. These data are interpreted as indicating that the assay method can not detect amounts of estrogen in milk of 0.09 μg/g or less.

Assay of milk from nonpregnant cows. To determine whether assayable amounts of estrogen are present in the milk of nonpregnant cows, milk samples were collected, dried, and assayed. The group of 11 cows of the station herd was in various stages of lactation, but had not been rebred (Table 3). Whether any

TABLE 3

Assay of milk from nonpregnant cows

Cow No.	Breed	Milk yield	Assay data: Mice	Body wt.	Uterine wt.	Uterine wt. / Body wt.
		(lb/day)	*(No.)*	*(g.)*	*(mg.)*	*(%)*
400	Holstein	55.4	10	17.4	7.84	0.05
314	Holstein	41.5	10	19.8	11.41	0.06
397	Holstein	32.9	10	21.3	10.60	0.05
429	Holstein	49.3	10	19.3	11.78	0.06
9	Brown Swiss	26.9	10	21.8	9.68	0.05
433	Holstein	58.8	10	21.3	10.08	0.05
337	Holstein	57.7	10	19.1	9.36	0.05
587	Jersey	38.0	10	22.8	10.07	0.05
417	Holstein	49.8	10	19.2	12.00	0.06 (Pasture)
3	Brown Swiss	47.0	8	18.8	10.24	0.06 (Pasture)
376	Holstein	66.8	8	20.1	12.17	0.06 (Pasture)

of the cows were in estrus during the milk-collection periods was not determined. It will be noted that three cows were on pasture when samples were collected. Cows of the Brown Swiss, Holstein, and Jersey breeds, varying in production from 26.9 to 66.8 lb. per day, were included. Milk from two of the cows on pasture produced uterine weights of 12 mg. or more. The nine other cows ranged from a low of 7.84 to 11.78 mg. The average uterine weight of the group of 11 milk samples was 10.48 mg., and uterine weight/% of body weight was 0.05. Since these uterine weights are within the range of the control milk samples, it is concluded that the milk of nonpregnant cows either is free of estrogen or contains amounts of estrogen which can not be detected by the assay method employed. It is of interest that two cows on pasture produced milk showing the highest uterine weights. Whether plant estrogens may have contributed to their response is not known.

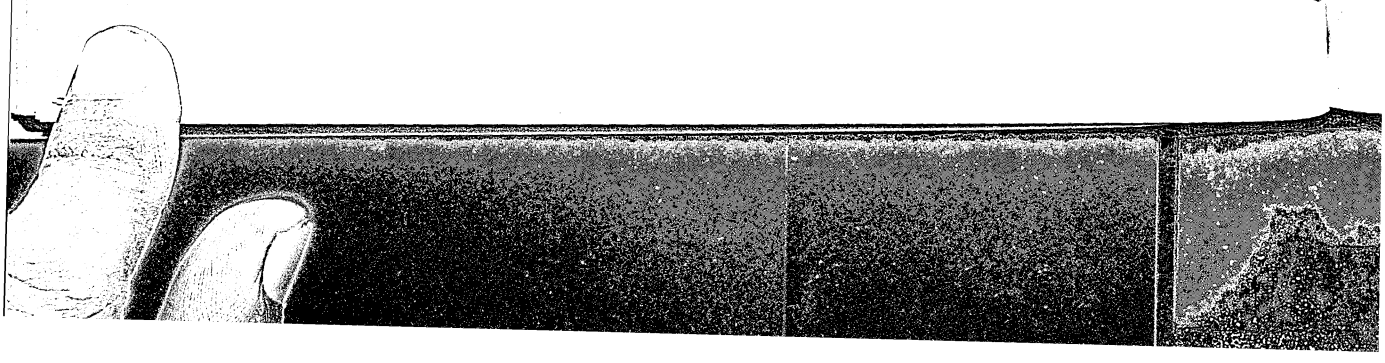
Assay of milk from pregnant cows. Milk from cows pregnant for various periods was collected, dried, and assayed as described. These cows were in various stages of lactation. The cows pregnant for the longer periods were usually

TABLE 4

Assay of milk from pregnant cows

Cow No.	Breed	Duration of pregnancy	Milk yield	Assay data			
				Mice	Body wt.	Uterine wt.	Uterine wt. / Body wt.
		(days)	*(lb/day)*	*(No.)*	*(g.)*	*(mg.)*	*(%)*
381	Holstein	27	16.9	10	21.6	10.56	0.05
611	Jersey	38	11.4	10	20.6	11.28	0.06
591	Jersey	45	13.7	10	18.6	11.12	0.06
443	Holstein	60	19.1	10	22.2	11.02	0.05
171	Holstein	70	35.0	10	23.8	11.10	0.05
600	Jersey	70	19.1	10	19.8	10.48	0.05
592	Jersey	77	24.0	10	19.2	11.12	0.06[a]
160	Jersey	82	17.9	10	20.4	14.73	0.07
420	Holstein	91	21.9	10	23.1	11.24	0.05
1	Brown Swiss	97	46.1	10	20.8	10.87	0.05
411	Holstein	104	26.8	9	16.9	8.73	0.05
590	Jersey	111	16.0	10	19.9	11.48	0.06
603	Jersey	152	24.6	10	20.5	11.44	0.06
370	Holstein	155	24.8	8	20.8	11.11	0.04
6	Brown Swiss	158	23.4	9	20.0	19.95	0.10
587	Jersey	160	9.3	11	20.8	13.70	0.07
436	Holstein	170	15.4	10	20.8	21.60	0.10
407	Holstein	178	21.9	10	19.1	11.71	0.06
8	Brown Swiss	181	19.8	10	19.3	17.81	0.09
598	Jersey	184	12.4	8	22.6	11.83	0.05
438	Holstein	193	12.4	10	17.9	11.48	0.06
434	Holstein	200	9.3	11	19.4	20.38	0.11
351	Holstein	206	23.4	7	18.4	11.14	0.04
7	Brown Swiss	207	14.3	9	20.1	19.05	0.10
417	Holstein	208	13.3	10	19.6	10.25	0.05
433	Holstein	210	15.5	9	18.0	16.56	0.09
346	Holstein	219	12.9	10	17.7	11.32	0.07
612	Jersey	222	7.2	9	18.1	43.53	0.24
304	Holstein	231	11.2	10	18.9	10.04	0.05
3	Brown Swiss	233	7.7	10	20.3	13.06	0.07
160	Jersey	238	7.1	8	17.3	13.38	0.08
160	Jersey	268	6.8	10	19.4	12.22	0.06

[a] Pasture eight days.



approaching the end of the lactation period and were producing only relatively small amounts of milk. The daily milk yield at the time the milk was collected is indicated in each case (Table 4).

The milk of ten cows from 27 to 97 days pregnant produced average mouse uterine weights of 11.35 mg., with an average uterine weight/% body weight of 0.06. Only a single cow in this group produced milk which stimulated uterine weight in excess of the control range.

The milk of 11 cows from 104 to 193 days pregnant produced average uterine weights of 13.71 mg., with an average uterine weight/% body weight of 0.068. In this group were three cows, each of whose milk was estimated to contain between 0.14 and 0.18 μg/g diethylstilbestrol-equivalent. The milk of the other cows was within the normal range.

The milk of 11 cows from 200 to 268 days pregnant produced average uterine weights of 16.45 mg., with an average uterine weight/% body weight of 0.09. Milk from one cow 222 days pregnant produced the greatest uterine response, 43.53 mg. The milk of three other cows in this group ranged from 16 to 20 mg., indicating a diethylstilbestrol response equivalent to about 0.18 μg/g dried milk. Four cows ranging from 222 to 268 days pregnant secreted milk producing uterine weights ranging from 10 to 13 mg.

In a concurrent study of the total estrogen excretion in the urine and feces of pregnant cows *(3)*, it was shown that low levels of estradiol-equivalent (4-5 mg/day) are produced during the first 160 days of pregnancy. Then there is a rapid increase in estrogen excretion to a level of about 20 mg/day, from 200 days to term. Although there is considerable variability in estrogen excretion in individual cows, the fourfold increase in estrogen during the latter part of pregnancy (200-280 days) might well have been expected to be reflected in an increased estrogen content of milk during this period. There was a slight average increase in uterine weight in this group, but it was owing, to a considerable extent, to a single milk sample.

These data on the estrogen content of the milk of pregnant cows are interpreted as indicating that the normal functioning mammary gland epithelial cells either are not permeable or are only slightly permeable to the normal circulating estrogens of the blood even in late pregnancy, when the estrogen secretion rate is high and the secretion of milk is low.

Assay of commercial market milk. To determine the amount of natural estrogens in commercial market milk, samples of processed milk from the University dairy sales room were obtained at monthly intervals, dried, and assayed. This milk was produced by the herds of the University at Columbia and represented milk from cows in all stages of lactation and pregnancy.

The first two samples obtained in August and September (1955), when the cows were on pasture, showed evidence of estrogen stimulation (Table 5). It was thought that the estrogen activity might have been due to the transfer of plant estrogens into the milk. The following spring, when the cows again were turned on pasture, a slight increase in estrogen activity in the milk again was observed one month, but was only slightly above the normal range. During the

TABLE 5
Assay of commercial milk for estrogen

Date of collection	Mice	Body wt.	Uterine weight	Uterine wt. / Body weight
1955	*(No.)*	*(g.)*	*(mg.)*	*(%)*
August 8	9	16.4	17.1	0.10
September 15	8	18.1	18.3	0.10
December 23	10	18.8	10.6	0.06
1956				
January 25	10	22.3	10.7	0.05
February 27	10	16.9	10.1	0.06
March 20	10	22.0	11.5	0.05
April 23	10	20.5	10.1	0.05
May 21	10	19.0	13.6	0.07[a]
June 18	10	18.4	10.9	0.06[b]
July 20	10	21.2	11.2	0.05
August 20	9	20.4	11.3	0.06
September 20	10	21.8	10.5	0.05
October 20	6	21.3	9.4	0.05
November 20	10	23.6	9.5	0.04
December 20	10	22.3	9.7	0.04
1957				
January 20	10	23.6	10.2	0.04
February 20	9	20.9	9.4	0.05
March 20	9	23.6	9.7	0.04

[a] Pasture 21 days.
[b] Pasture.

summer and fall, no further evidence of increased estrogenic activity was observed.

During the period of the 18 mo. that the samples of milk were assayed, the uterine response varied from 18.3 to 9.41 mg., with an average of 11.32 mg., and uterine weight/% of body weight of 0.06. These data lend further support to the thesis that estrogenic hormones are only slightly, if at all, permeable to the mammary gland epithelium.

Estrogen in colostrum and early lactation. Samples of colostrum and milk obtained at 10, 30, 60, and 90 days following parturition have been obtained from four individual cows and at ten to 30 days in two additional animals (Table 6). These data show that the colostrum (first milk) contains very little estrogen, the uterine weights ranging from 11.46 to 14.16 mg., with an average of 12.70 mg. and uterine weight/% of body weight of 0.06. This is very remarkable, because at this time the amount of circulating blood estrogen is at a peak (at time of parturition) and the functional activity of the mammary glands cells is low. Further, the fact that the cells at this time are especially permeable to blood globulins, which are in high concentration in colostrum, might lead one to expect high estrogen permeability at this time. Estrogens in circulating blood are believed to be combined loosely with blood protein (estroprotein), which might increase their permeability to the cell. Apparently, this binding is not sufficient to permit the passage of estroprotein into the colostrum in appreciable amounts.

With the advance in lactation, there appears to be a tendency for the uterine

TABLE 6

Estrogen content of colostrum and milk from early lactation

Cow No.	Breed	Remarks	Assay data			
			Mice	Body weight	Uterine weight	Uterine wt. / Body wt.
			(No.)	*(g.)*	*(mg.)*	*(%)*
402	Holstein	Colostrum	7	20.9	14.16	0.07
		10 days	9	23.0	12.80	0.05
		30 days	9	19.7	38.31	0.20
		60 days	9	17.7	10.14	0.06
		90 days	10	19.8	10.57	0.05
160	Jersey	Colostrum	10	19.6	13.85	0.07
		10 days	10	21.6	11.46	0.05
		30 days	10	19.4	9.19	0.05
		60 days	10	21.3	17.25	0.08
		90 days	10	21.0	8.53	0.04
583	Jersey	Colostrum	9	19.2	13.13	0.07
		10 days	10	24.7	10.17	0.04
		30 days	10	22.8	10.11	0.05
		60 days	10	19.1	9.83	0.05
		90 days	10	18.1	8.15	0.05
159	Jersey	Colostrum	10	22.0	11.77	0.06
		10 days	10	26.8	10.90	0.04
		30 days	9	20.5	8.74	0.04
		60 days	10	19.9	8.65	0.04
		90 days	10	22.0	8.91	0.04
585	Jersey	Colostrum	10	20.7	11.46	0.06
		10 days	10	20.3	9.13	0.05
		30 days	10	21.5	11.11	0.05
601	Jersey	Colostrum	10	21.8	11.85	0.06
		10 days	10	21.2	9.52	0.05
		Lactation experimentally induced				
594	Jersey	Colostrum	8	17.4	11.69	0.07
		10 days	9	19.4	13.59	0.07
		30 days	10	19.8	9.20	0.05
		60 days	10	19.5	9.40	0.05
		91 days	6	21.3	9.60	0.05
3625	Guernsey	Colostrum	10	19.5	9.88	0.05
18	Guernsey (Freemartin)	Colostrum	10	22.2	11.64	0.05

weights of the assay animals to decline slightly, although an increased uterine response was observed in milk samples from two animals at 30 and 60 days of lactation. No explanation can be offered for these higher responses.

It is believed that these observations are most critical in regard to the permeability of estrogen to the mammary gland epithelial cells, because the secretion of estrogen is at a maximum (as indicated by estimations of estrogen in the urine and feces in late pregnancy) and the rate of milk secretion (colostrum) in late pregnancy is minimal.

Effect of feeding diethylstilbestrol upon the estrogen content of milk. It was shown in our laboratory that the injection of estrogen into experimental animals stimulated an increased secretion of the lactogenic hormone. It has been suggested that the initiation of lactation in normal pregnant animals at the approach of parturition is owing to an increased secretion of estrogen *(7, 8)* which, in turn,

stimulates the increased secretion of the lactogenic hormone. Based on this theory, estrogen was administered to the experimental sterile heifers after udder growth had been produced. That estrogen is effective in initiating lactation at this time has been demonstrated *(14)*.

In the sterile heifers induced to lactate by this method, there was a gradual increase in milk yield, reaching a peak, then a gradual decline, similar to the decline observed in normal lactating cows. In a group of these heifers, the feeding of 10 mg. of diethylstilbestrol daily tended to prevent the decline in lactation observed. It was suggested that the feeding of estrogen might be an effective method of maintaining a higher level of secretion of the lactogenic hormone. The data presented *(14)* indicated such a role.

The problem arose as to whether the feeding of diethylstilbestrol or other synthetic estrogens to cows, after lactation started to decline, would increase the estrogen content of the milk. Whereas the previous results indicated that the natural estrogens are either nonpermeable or only slightly permeable to the lactating epithelial cell, within the normal range of circulating estrogens during pregnancy, there might be a difference in the permeability of the gland cells to the synthetic estrogens such as diethylstilbestrol.

A group of nonpregnant cows, ranging in production from 5.4 to 26.8 lb. per day, was fed diethylstilbestrol at levels varying from 10 to 20 mg. per day for periods of 2 wk. or more. Lower-producing cows were used, in order to accentuate the concentration of the estrogen if the cells showed indications of permeability to the hormone. The nine milk samples from cows fed 10 mg/day showed a range in uterine weight from 17.56 to 7.67 mg., with an average of 12.07 mg., and an average uterine weight/% body weight of 0.07. The two samples from cows fed 15 mg/day averaged 9.65 mg., whereas one sample from a cow fed 20 mg/day produced an average uterine weight of 15.44 mg. (Table 7).

These milk samples, considered as a group, show minimal stimulation of the

TABLE 7

Diethylstilbestrol feeding on estrogen content of milk

Cow No.	Breed	Milk yield	Remarks	Assay data			
				Mice	Body weight	Uterine weight	Uterine wt. / Body wt.
		(lb/day)	*(mg.)*	*(No.)*	*(g.)*	*(mg.)*	*(%)*
310	Jersey	15.0	10 [a]	10	15.1	11.04	0.07
580	Jersey	22.1	10 [a]	10	15.9	13.98	0.09
		15.4	10	10	19.8	10.30	0.06
585	Jersey	12.6	10	10	21.8	11.68	0.05
		26.8	15	10	23.9	10.00	0.04
544	Jersey	13.5	10	9	13.0	17.08	0.14
		17.2	10	10	17.9	17.56	0.10
		18.1	20	10	24.1	15.44	0.07
		12.8	10	10	20.4	9.36	0.05
594	Jersey	9.8	10	9	18.2	7.67	0.04
601	Jersey	28.4	10	10	23.1	9.94	0.04
3596	Guernsey	5.4	15	10	19.4	9.30	0.05

[a] 90% Dried milk + 10% mouse feed.

uterus. The samples showing the greatest estrogenic activity indicate concentrations of diethylstilbestrol equivalent to 0.14 μg/g of dried milk, which is comparable to some samples of milk from cows in late pregnancy.

From these data, it is concluded that the feeding of diethylstilbestrol at levels of from 10 to 20 mg/day to lactating cows permits the passage into the milk of either no estrogenic hormone or minimal amounts of it.

Estrogen assay of milk from cows experimentally induced to lactate. A number of sterile heifers have been treated with estrogen and progesterone for a period of 180 days, to stimulate the growth of the udder. At the end of this time, the treatment was stopped and lactation was initiated by the daily injection of 3 mg. of estradiol benzoate, for a period of 14 days. Milking was started at this time. The yield of milk was limited for a few days, then increased rapidly for a period of weeks *(14)*. To determine the amount of estrogen present in this first milk (colostrum?), samples from a Jersey and two Guernsey cows were dried and assayed. In the two Guernsey cows, only the colostrum was assayed; whereas, in the Jersey (No. 594) samples were obtained at intervals up to 90 days, as in the previous study. It will be noted that only minimal stimulation of the uterus was induced, comparable to the observations in the cows which calved normally (Table 6).

DISCUSSION

The present study was initiated to determine the extent to which the functional mammary gland serves as a normal pathway of excretion of estrogen in dairy cattle. In the human, as well as in cattle and some other domestic animals, lactation and pregnancy are concurrent. During pregnancy, the placental secretion of estrogen gradually increases, reaching a peak in late pregnancy. If the epithelial cells of the lactating mammary gland were permeable to estrogen, one might expect the presence of increasing amounts of estrogen in the milk secreted in late pregnancy and in the colostrum after parturition.

The observations pesented indicate clearly that such is not the case. Within the sensitivity of the assay method, milk is essentially free of estrogens even during periods of highest estrogen secretion and low milk secretion. The data showing the extremely low levels of estrogenic activity in colostrum, which is secreted at a time when the mammary cells are permeable to blood globulins and certain other blood constituents, are of special significance.

The discovery of the presence of isoflavones with estrogenic activity in clovers and soybean meal has raised the question of the possible presence of these compounds in milk. Although a slight increase in estrogenic activity was observed in some samples of milk from cows on pasture, significant levels of estrogenic activity which could be ascribed to plant sources are not believed to be present in milk.

The feeding of synthetic estrogens such as diethylstilbestrol to lactating dairy cattle, to stimulate the continued secretion of high levels of the lactogenic hormone and thus maintain intense milk secretion during the normally declining period of lactation, is believed to hold promise. The question of the permeability of the mammary gland epithelium to the synthetic estrogens is naturally raised.

The present study indicates that the levels of diethylstilbestrol, i.e., from 10 to 20 mg. per day to cows weighing about 1,000 lb., is not sufficient to permit the passage of significant amounts of estrogen into the milk. If estrogen is present in the milk, it is at the border line of sensitivity of the assay method employed. As a means of comparison, it should be pointed out that the samples showing the greatest estrogenic activity were estimated to show the diethylstilbestrol-equivalent of 0.14 μg/g dried milk. It has been shown that to stimulate the growth of the mammary gland duct system of male mice in a period of from 3 to 4 wk., it would require 1.23 μg/g of mouse feed *(2)*, or about ten times the amount estimated to be present in the milk.

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THYROIDAL UPTAKE OF I^{131} BY DAIRY CALVES [1]

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SUMMARY

This study shows some of the factors causing variation in the 48-hr. uptake of radioactive iodine in 83 dairy calves for 2 yr. The average uptake was 36.4% of the injected dose, with considerable variation within and between trials. The Brown Swiss calves averaged 10% lower than calves of the other breeds. The highest average per cent of uptake was found in the summer, and the lowest in the spring. Hourly counts calculated as the per cent of the 24-hr. count showed little difference in the rate of uptake between breeds. A statistical analysis of the uptake of 64 Brown Swiss, Holstein, and Jersey calves showed a significant difference due to breed, season, breed × season, and other interactions. The age differences were not significant. A correlation coefficient of −0.17 was found when 37 observations of the 48-hr. uptake and the estimated thyroxine secretion rate were analyzed. The addition of iodized salt to the ration, and injecting the isotope intravenously rather than subcutaneously, had no apparent effect on the percentage of uptake. From the findings in this study and those of other workers, it appears questionable whether the thyroidal uptake of I^{131} alone will provide worthwhile information on the thyroid status of dairy calves.

One of the methods used to measure changes in the activity of the thyroid is to determine the per cent of an injected dose of I^{131} collected by the thyroid over a period of time after injection of the isotope. Werner *et al. (9)* and others used the 24-hr. uptake of radioactive iodine as the most stable time at which to assay thyroid function in humans. The uptake of radioactive iodine has been used by Swanson *et al. (7, 8)* and by Blincoe and Brody *(2, 3)* as the basis for determining the effect of several factors on thyroid activity in dairy cows. However, it has not been demonstrated in the dairy animal that the uptake of radioactive iodine is a reliable measure of thyroid activity. This paper describes some of the factors which may affect the uptake of radioactive iodine by the thyroid gland of dairy calves, and also correlates some of these values with actual thyroid secretion rates.

EXPERIMENTAL PROCEDURE

The experimental animals were from the Michigan State University dairy herd and included calves of the Ayrshire, Brown Swiss, Guernsey, Holstein, and Jersey breeds. The average age was 5.2 mo., with a standard deviation of 2.2 mo. The calves received the regular rations used at the University which, in the early trials, did not include supplemental iodine. However, for the later trials the ration was supplemented with iodine by mixing 1% of iodized salt containing 0.02% potassium iodide into the concentrate.

A tracer dose of carrier-free I^{131}, ranging from 50 to 150 μc., was injected either subcutaneously or intravenously into each heifer. The uptake of I^{131} by

Received for publication September 3, 1957.

[1] Approved by the Director, Michigan Agricultural Experiment Station, as Journal Article No. 2125.

the thyroid gland was followed by taking counts of the radioactivity from the thyroid area of the neck at hourly, daily, or alternate-day intervals. As far as possible, the same amount of pressure was applied to the neck of the calf each time counts were taken. The percentage of uptake was calculated from the maximum repeatable count obtained from this region of the neck. Corrections were made for background and the physical decay of the isotope. A Co^{60} source was used to standardize the instruments.

During the earlier studies, a shielded end-window TGC-8 Geiger-Mueller Tube (Tracelab) was used with a Model 1615 B count-rate meter (Nuclear). A scintillation counter and Model 1620 count-rate meter (Nuclear) were used for the later work, allowing a reduction in the amount of isotope injected into each animal because of the greater sensitivity of the instruments.

Aliquots of the injection solution were counted in a "phantom neck," similar to that described by Blincoe *(1)* for use as a standard in determining the percentage of uptake.

A series of trials was conducted during a period of 2 yr., with eight calves being used in each trial.

RESULTS AND DISCUSSION

Usually, the highest degree of thyroid radioactivity was reached about 48 hr. after injection. However, some animals showed a maximum count on the first day and many animals continued to accumulate I^{131} for a week or more.

Considerable variation in the 48-hr. uptake of I^{131} was found within, as well as between, trials. Some of the factors causing these differences could have been age, breed, season, level of iodine in the ration, and route of administration of the isotope. From this study, the major factors involved appear to be breed and season. The average 48-hr. uptake of 83 calves was 36.4% of the injected dose. The average percentage of uptake according to breed is shown (Table 1). The largest difference was found between the Brown Swiss and the other breeds. There was considerable variation in uptake between individuals within a breed, but the Brown Swiss calves averaged about 10% lower in comparison with the average uptake of calves from the other breeds.

A large amount of variation in the 48-hr. uptake was noted during the course of the study for different months during the year. In order to make a comparison, the year was divided into the four seasons: Winter—January, February,

TABLE 1

Effect of breed on the 48-hr. uptake of I^{131} by the thyroid gland of dairy calves

Breed	No. of calves	Percentage uptake	Standard deviation
Ayrshire	3	44.7	.....
Brown Swiss	26	29.3	13.8
Guernsey	8	38.9	15.7
Holstein	22	37.9	13.6
Jersey	24	40.9	17.4
Average	83	36.4	

March; spring—April, May, June; summer—July, August, September, and fall—October, November, and December. The average uptake for the four seasons is shown (Table 2). The highest uptake occurred in the summer and the lowest in the spring. These seasonal differences in uptake are not surprising, in view of the observation of Seiden and Fenger *(6)* that the iodine content of cattle thyroids was highest in late summer and at a minimal level in the late winter. However, it is well known that in other species of animals thyroid secretion is lowest in the summer and highest in late winter. Although there are few data to show any seasonal differences in the thyroid secretion rate of dairy animals, there is no reason to believe it would be the reverse of other species. Therefore, it is believed that the iodine content of the thyroid gland is not indicative of the gland's activity but only shows its storage capacity. The same can be said for uptake of radioactive iodine, in that it is a measure of the collecting ability of the gland and, owing to storage of iodine, may not be indicative of the output of the gland. Based on the results of this study, the 48-hr. uptake indicates a period of high storage of radioactive iodine in the summer and a period of low storage in the spring.

So few Ayrshires and Guernseys were studied that an adequate comparison of all breeds could not be made. However, a statistical analysis was made to determine the age, season, and breed differences in the percentage of uptake of I^{131} of 64 Brown Swiss, Holstein, and Jersey calves. Because of the distribution of the calves in each classification, a method of least squares was used to determine the effect of each component. Distribution of the animals according to breed and season, and the variation in the 48-hr. uptake, are shown (Table 3). The ages of the 64 calves included in the analysis ranged from 3 to 9 mo. To determine the variation due to season, the year was divided into the four seasons, as previously described.

TABLE 2

Effect of season on the 48-hr. uptake of I^{131} by the thyroid gland of dairy calves

Season	No. of calves	Percentage uptake	Standard deviation
Winter—(January–March)	24	38.4	9.7
Spring—(April–June)	24	26.7	10.4
Summer—(July–September)	12	53.6	18.5
Fall—(October–December)	23	35.5	15.5

TABLE 3

Breed and seasonal variation in 48-hr. uptake of I^{131}

Breed	Winter		Spring		Summer		Fall	
	No.	Uptake	No.	Uptake	No.	Uptake	No.	Uptake
		(%)		*(%)*		*(%)*		*(%)*
Brown Swiss	4	31.2	9	20.5	5	48.5	5	26.0
Jersey	7	40.6	8	23.6	3	70.3	7	44.6
Holstein	5	40.8	4	40.6	4	47.2	3	24.8

The component effects of age, breed, and season, as determined by method of least squares, are shown (Table 4) by the deviation from the over-all average; the analysis of variance also is shown (Table 5).

Results of the analysis show a significant difference due to breed, season, breed × season interaction, and the other interactions. The age differences were not significant, which is what one might expect since the range in ages was not large. However, there is an indication that the younger calves had a higher average uptake than did the older calves. The most important finding from the analysis was the highly significant breed × season interaction. This interaction, along with the breed or season differences, further complicates the use of the uptake of radioactive iodine in making comparisons of dairy calves because, when making a breed or season comparison, one has to specify the particular breed and season being compared. The other interactions could not be divided into individual component parts because of the distribution of the animals, but their significance is probably due to the breed and season differences.

TABLE 4

Component effects shown by deviation from the average 48-hr. uptake of I^{131}

General effects	Deviation from av.[a]	Interactions	Deviation from av.[a]
Age (mo.)			
3	7	Winter ×	
4	6	Brown Swiss	− 2
5	10	Jersey	2
6	− 4	Holstein	0
7	− 4		
8	− 5	Spring ×	
9	− 9	Brown Swiss	1
		Jersey	−11
Breed		Holstein	10
Brown Swiss	− 9	Summer ×	
Jersey	8	Brown Swiss	1
Holstein	0	Jersey	7
Season		Holstein	− 8
Winter	0	Fall ×	
Spring	−12	Brown Swiss	0
Summer	13	Jersey	2
Fall	− 1	Holstein	− 2

[a] Average = 35.

TABLE 5

Analysis of variance

Source	d.f.	s.s.	m.s.
Age	6	1,171	195
Breed	2	2,426	1,213[a]
Season	3	4,265	1,422[a]
Breed × season	6	1,652	275[b]
Other interactions	24	3,590	150[a]
Within cells	22	1,601	73

[a] Significant at 5%.
[b] Significant at 1%.

Since Michigan lies in a goitrous area, and the calf rations in the earlier trials did not include supplemental iodine, it was thought that the addition of supplemental iodine to the ration might have an effect on the uptake of I^{131}. About midway in the study, iodized salt containing 0.02% potassium iodide was mixed in the concentrate ration at the level of 1%. The addition of the supplemental iodine, at the level added, had no apparent effect on the uptake of I^{131} by the thyroid gland of calves, when compared with trials conducted in the same season the previous year, when no supplemental iodine was included. However, the amount of iodine administered might not have been in excess of the animals' needs, and further study needs to be done to determine the effect of an excess of iodine on the uptake.

It was also thought that intravenous injection of the isotope might possibly have an effect on the 48-hr. uptake, compared to subcutaneous injection. To determine this, the calves in three trials were injected intravenously in the jugular vein. No difference was found in the percentage of the injected I^{131} collected by the thyroid gland in 48 hr. by this route of injection, when compared with the uptake by calves injected subcutaneously.

Hourly counts for a period of 8 hr. were obtained from the calves receiving the intravenous injections, to show the rate of uptake of the isotope. The rate of uptake was calculated as the per cent of the 24- or 48-hr. count. The per cent of 24-hr. count for the first 8 hr. of 24 calves is shown (Table 6). When the per cent of the 24- or 48-hr. count is plotted, the accumulation of I^{131} for the total period of time is curvilinear, but it may approach linearity over a small segment of time, particularly during the first few hours after injection. Even though there was a difference between breeds, in the per cent of the injected dose collected up to 48 hr., when calculated in the manner above, there does not appear to be a large difference in the rate of uptake. The percentage increase for each hour, except for the first hour, shows that the thyroid gland of the Brown Swiss calves collected I^{131} at about the same rate as did the calves of the other breeds.

TABLE 6

Hourly uptake of I^{131} expressed as percentage of 24 hr.

Breed	No. of calves	Percentage by hour						
		1	2	3	5	6	7	8
Holstein	10	11.4	17.7	22.4	33.3	36.8	40.0	46.5
Brown Swiss	8	6.1	11.0	15.8	25.4	29.4	30.9	38.4
Jersey	4	8.3	15.1	19.8	29.4	35.0	41.0	44.3
Guernsey	2	10.2	16.0	22.7	34.4	38.3	41.8	.....
Average		9.0	14.9	19.8	30.1	33.9	37.5	43.4

The present authors have reported a technique for estimating the daily thyroxine secretion rate of dairy calves *(5)*. It is the best method available at the present time for determining the actual output of the thyroid hormone which, without question, is the best criterion for measuring gland activity. A correlation analysis was run on 37 observations of the 48-hr. uptake of I^{131} and the

estimated thyroxine secretion rate. The correlation coefficient of −0.17 was not statistically significant. However, there appears to be a tendency toward an inverse relationship between the 48-hr. uptake and the thyroxine secretion rate, as found in this study. This same relationship has been reported for mice by Chai *et al. (4)*. In the light of this study, as well as the findings cited earlier, it appears extremely questionable whether thyroidal I^{131} uptake alone will provide worthwhile information on the thyroid status of dairy calves.

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EFFECT OF HAY-CONCENTRATE RATIO ON RUMEN FILL AS MEASURED BY AN ANTIPYRINE DILUTION TECHNIQUE [1]

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SUMMARY

The rumen contents of seven fistulated bovines were removed and weighed at from 2 to 24 hr. after feeding. These 477- to 1,310-lb. animals were fed from 1.3 to 3.1 lb. of air-dry material per 100 lb. body weight per day in one portion. Regression equations were developed for the total contents and water contained in the reticulo-rumen as a function of the per cent concentrate in the ration, time after feeding, and plane of nutrition. The per cent concentrate in the ration had a much larger effect on the amount of rumen contents than either time after feeding or plane of nutrition. Hay-concentrate ratios did not significantly affect dry matter concentrations in the rumen. The dry matter content of rumen ingesta decreased with time after feeding ($P < 0.01$). An antipyrine dilution method was developed for estimation of the water content of the reticulo-rumen. The regression equations were found to give a better estimate than the antipyrine dilution values.

The proportion of the body weight which is constituted by the contents of the forestomachs has been of interest in research on body composition *(7)*, appetite *(6)*, and rumen fermentation *(3, 4)*. The mass of the reticulo-rumen contents is of direct economic importance as a factor in the shrinkage or fill involved in marketing cattle *(1)*. Recent reviews indicate that considerable variability exists in the mass of the reticulo-rumen contents, but they give little information concerning the cause *(3, 6)*. The present study was undertaken to study these factors and to develop a simple method for measuring the mass contained in the reticulo-rumen of living cattle.

METHODS AND PROCEDURES

The four cows, one heifer, and three steers used in this study were equipped with the plastic fistula plug of Hentschl *et al. (5)*. The cows were dry, but represented all stages of pregnancy. All animals were fed from 1.3 to 3.1 lb. of air-dry material per 100 lb. of body weight once daily at 8 A.M. Body weights ranged from 477 to 1,310 lb. The contents of the reticulo-rumen were removed, weighed, and sampled at various times between 2 and 24 hr. after feeding. Measurements were made after a 3-wk. stabilization period on each ration. The rations contained from 0 to 80% concentrate. The concentrate varied slightly, but was based on a mixture of four parts ground corn to one part of soybean oil meal. The remainder of the ration was alfalfa or alfalfa-grass hay which varied greatly in quality and stage of maturity.

The water content of the rumen was also determined by mixing with the fist from 10 to 50 ml. of 10% antipyrine solution throughout the rumen contents for

Received for publication August 20, 1957.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2120.

a period of 2 min. Mixing was repeated after a 5-min. interval, and a sample withdrawn. This sample was then centrifuged at 14,000 r.p.m. for 20 min. and the antipyrine concentration in the supernatant determined by the chloroform extraction procedure of Brodie *et al. (2)*.

RESULTS AND DISCUSSION

The total contents and the water of the reticulo-rumen, as determined by removal of these contents, have been fitted to a multiple regression equation as described by Snedecor *(8)*. A total of 42 determinations are included:

Y = Total reticulo-rumen contents expressed as per cent of body weight

W = Amount of water in the reticulo-rumen expressed as per cent of body weight

X_1 = Per cent concentrate in the ration

X_2 = Time after feeding, in hours

X_3 = Plane of nutrition expressed as lb. of air-dry feed consumed per 100 lb. body weight.

$$Y = 12.21 - 0.05\ X_1 - 0.13\ X_2 + 2.65\ X_3$$
$$W = 11.85 - 0.05\ X_1 - 0.10\ X_2 + 1.78\ X_3$$

The standard errors of estimate for Y and W, respectively, are 2.72 and 2.82. Expression of Y and W as per cent of body weight implies an assumption of a linear relationship between these variables. The small standard errors of Y and W found in this study, involving a wide range of body weights, lend support to this assumption. Standard errors for the regression coefficients for X_1, X_2, and X_3, respectively, are about 0.01, 0.03, and 0.7. These relationships also are shown (Figure 1).

The regression coefficients for X_2, time after feeding, show that the total ingesta decreases faster than the water in the reticulo-rumen ($P < 0.01$). Thus, the dry matter content of rumen ingesta decreases with time after feeding when the amount and quality of the ration are constant. This is in agreement with Mäkalä *(6)*. A third regression equation for the dry matter of the rumen can be obtained by substracting W from Y. Dry matter concentration in the reticulo-rumen was not significantly affected by the hay: concentrate ratio, but did tend to increase with increasing plane of nutrition.

It has been assumed that Y and W vary in a linear manner with each of the X's. This is true for X_1 in this study, but the data are not adequate to test linearity with X_2 and X_3.

Highly significant ($P < 0.01$) multiple correlation coefficients of 0.70 and 0.64 were found for Y and W. It should be noted that around 30% of the variability remains to be accounted for. Factors which remain to be considered are quality of hay, stage of pregnancy, short- and long-term previous environment, degree of fatness, and inheritance. Quality of the forage probably is particularly important. Balch and Line *(1)* have observed 20 to 30% decreases in the mass of

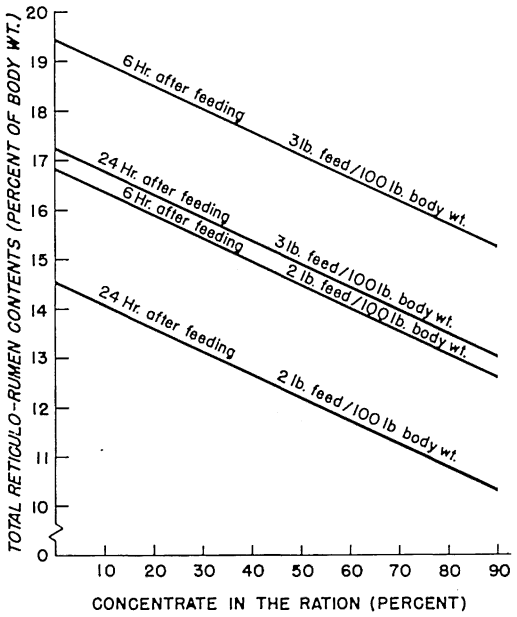


FIG. 1. Rumen fill as calculated from the regression equation.

the reticulo-rumen contents with a change from dry-feeding to pasture. Also, Stallcup *et al. (9)* reported that retention time of hays in the reticulo-rumen increases with increasing lignin content. Observations should be made on green forage and silage, also.

The standard partial regression coefficients (Table 1) are independent of units; thus, they express the relative contribution of X_1, X_2, and X_3 to Y and W. When each standard partial regression coefficient was divided by the sum of the 3, it was found that ration accounted for 44% of the variation considered in this study, whereas time after feeding accounted for 30%, and plane of nutrition, 26%.

TABLE 1

Standard partial regression coefficients and correlation coefficients

	X_1	X_2	X_3
Correlation between Y and X's	−0.55	−0.35	0.53
Correlation between W and X's	−0.55	−0.28	0.45
Standard regression coefficients of Y on X's	−0.41	−0.33	0.30
Standard regression coefficients of W on X's	−0.45	−0.27	0.21

The water content of the reticulo-rumen, as determined by the antipyrine dilution method just described, yielded values $131.6 \pm 7.9\%$ of the values determined by emptying the rumen. When the antipyrine was allowed to mix with the rumen contents naturally, distribution remained uneven for as long as 1 hr. Disappearance of antipyrine from the reticulo-rumen was uneven and unpredictable. The regression equations will give a better estimate than the antipyrine dilution value. The N-acetyl-4-aminoantipyrine method of Reid *et al. (7)* should be more suitable.

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COMPARISON OF IN VIVO AND IN VITRO TECHNIQUES IN RUMINOLOGY STUDIES [1]

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SUMMARY

This paper is concerned with the comparison of in vivo and in vitro rumen bacterial dissimilations of some carbohydrates, amino acids, and organic acids of metabolic importance. It was observed that succinate, DL-aspartate, glucose, and maltose were readily fermented by rumen microorganisms, and that volatile fatty acids (VFA) were produced. The amino acids DL-lysine, DL-alanine, and glycine were not degraded to an appreciable extent to VFA by rumen bacteria. In general, qualitative agreement between in vivo and in vitro washed cell suspension (WCS) experiments was obtained with DL-lysine, DL-alanine, and glycine, while the in vivo studies using DL-aspartate and succinate agreed with the WCS in vitro studies of Sirotnak *et al. (17, 18)*. With the substrates glucose and maltose, in vitro WCS experiments were both variable and in disagreement with the in vivo studies. Thus, the WCS technique appears most useful for studying short one- or two-step reactions presumed to occur in the rumen. This technique loses significance when studying multistep metabolic reactions and, in such cases, the results should be interpreted with caution until they are verified by in vivo experiments, or until further studies concerning the limitations of the WCS technique are available.

In vitro investigations of the rumen flora require the use of one, or a combination, of the following methods: Microscopic examinations, pure culture studies of bacteria, studies on cell-free enzymes from bacterial or protozoan suspensions, and various types of the so-called artificial rumen *(3)*. Because the determination of volatile fatty acids (VFA) in the rumen is, at best, only a rough estimate of production and can not be used for precise studies on the metabolism of rumen bacteria, Johns *et al. (9)*, Sijpesteijn and Elsden *(16)*, and later, Doetsch *et al. (5)* have used buffered washed cell suspensions (WCS) of rumen bacteria in an attempt to add percision to such studies. Elsden *(16)* stated the following advantages for this technique: *(a)* The population is studied as an entity under controlled conditions; *(b)* the enzymatic action of the population in the absence of an added substrate is negligible; *(c)* since the experimental period is short and growth is not permitted, the chance of significant alteration in the composition of the population is nil, and *(d)* small-scale work is possible which permits the investigation of the degradation of expensive or toxic materials. This technique and its modifications have been described by Doetsch *et al. (5)*.

An extensive study was conducted by Doetsch *et al. (4)*, in which the washed cell suspension technique was used to study catabolic reactions of rumen bacteria. The dissimilation of numerous substrates was studied in regard to gas and VFA production. Similar studies on amino acids, polysaccharide synthesis, and lactic

Received for publication August 6, 1957.

[1] Scientific Article No. A641, Contribution No. 2824 of the Maryland Agricultural Experiment Station.

acid formation were reported by Sirotnak *et al. (17)*, Gibbons *et al. (8)*, and Jurtshuk and Hueter *(10)*. A later study was conducted by Sirotnak *et al. (18)* on aspartate dissimilation reactions of rumen bacteria. The results confirmed previous findings and, in addition, indicated the probable pathways of dissimilation of aspartic acid.

The data reported in this paper are concerned with the comparison of in vivo and in vitro rumen bacterial dissimilations of some carbohydrates, amino acids, and organic acids of metabolic importance. The purpose was to determine the extent to which in vitro dissimilations are representative of in vivo occurrences. Our intention was to make a simultaneous and more direct comparison between in vivo and in vitro results than has been done heretofore.

EXPERIMENTAL PROCEDURE

Animal feeding. Two permanently fistulated, nonlactating and nonpregnant cows (Jersey and Holstein) were used as experimental animals. They were fed slightly in excess of Morrison's recommended standards *(14)* on a ration of 8 lb. of alfalfa hay and 6 lb. of 16% protein concentrate mixture. The concentrate ration was fed twice daily except on days of experimental trials, when the morning feeding was omitted. Hay was fed only in the evening, and in controlled amounts to insure complete consumption by 12 midnight preceding a trial. On trial days, water was withheld from the animals starting at 12 midnight and ending at 4:30 A.M., at which time they were allowed access to water for approximately 0.5 hr. The source of water was then removed, and to afford sufficient time for the ingested water to reach equilibrium within the animal, an interval of approximately 3.5 hr. was allowed before the start of the trial. After the morning watering, the animals did not receive water again until the end of the trial period. The cows remained at approximately constant weight and were in good health throughout the entire study.

Sampling. Rumen liquor samples (500 ml.) were obtained via the fistula with the aid of a metal cannula and rubber hose connected to an aspirator pump. Samples were taken prior to administration of substrate and at intervals after substrate addition. Immediately after removal, the rumen liquor was strained through two layers of cheesecloth, then centrifuged at 1,000 × G for 15 min. to remove the residual feed particles. Aliquot samples of the resultant supernatant liquid were preserved for lactic acid and volatile fatty acid analyses. Samples for lactic acid determination were preserved by adding 0.1 ml. of 100% (w/v) trichloroacetic acid to 1 ml. of rumen liquor. Samples for VFA determinations were preserved by adding 1 ml. of a saturated mecuric chloride solution to 9 ml. of rumen liquor.

The pH of rumen samples, when determined, was measured within 2 to 3 min. after removal. Although these measurements probably did not represent the precise intraruminal pH, it appears reasonable to assume that any large changes in rumen liquor pH would be detected by this procedure.

For in vitro dissimilation studies, cell suspensions were prepared as described by Doetsch *et al. (5)*. These cell suspensions were made from the rumen liquor

of an experimental animal, on which an in vivo study of the same substrate was being made simultaneously. Two different cell suspension dissimilations were performed for each in vitro study. One cell suspension, referred to hereafter as the 0-hr. suspension, was prepared immediately before adding the substrate to the rumen. The other cell suspension, referred to hereafter as the 5-hr. suspension, was prepared from the rumen liquor of the same cow 5 hr. after the administration to the rumen of the substrate being studied. The bacteria in the 0-hr. suspension had no previous contact with the substrate, whereas those of the 5-hr. suspension were exposed for 5 hr. to the particular substrate being investigated. The fermentation liquor of in vitro dissimilation studies was saved for VFA determinations. The samples were preserved as just described.

Rumen liquor lactic acid was determined by the method of Barker and Summerson *(1)* and the VFA by the method of Keeney *(11)*. The Beckman pH Meter, Model H-2, using standard glass electrodes, was used for measuring the pH of rumen liquor.

All substrates used in the in vivo studies were diluted with water to a volume of 1.5 to 2.0 liters, then administered via the rumen fistula. The following substrates were used for in vivo studies: sodium succinate, DL-aspartic acid, DL-aspartic acid neutralized to pH 6.9 with Na_2CO_3, D-glucose, maltose, DL-lysine, and a mixture of glycine plus DL-alanine. In order to compare in vivo with in vitro results, in vitro dissimilations were made on the same substrates, with the exception of succinate and aspartate. The dissimilation of these latter substrates by WCS has been studied in detail by Sirotnak *et al. (17, 18)*; hence, was not included.

RESULTS

Sodium succinate and DL-*aspartic acid.* Results of in vivo studies with sodium succinate and DL-aspartic acid are summarized (Table 1). As a control, the rumen liquor analyses for a cow from which feed had been withheld for 18 hr. preceding the sampling period are shown. It will be noted that the concentration of propionic acid in the rumen increased approximately 17 and 9 molar per cent, respectively, following additions of sodium succinate and neutralized DL-aspartic acid. Decreases occurred in the rumen liquor concentrations of acetic acid and butyric plus higher acid fractions following the administration of sodium succinate. A decrease also occurred in the acetic acid concentration of the rumen liquor of the cow which received neutralized DL-aspartic acid, with a concurrent decrease in butyric acid that appeared almost equal to the increases in valeric and higher acids. This increase in the valeric and higher acid fraction may have been owing to an increase in branched-chain fatty acids resulting from the Stickland-type reaction, as suggested by El-Shazly *(7)*.

The DL-aspartic acid which was not neutralized was not dissimilated to VFA. Indeed, the concentration of total VFA decreased more rapidly than in the fasting cow, probably owing to the low pH of the rumen liquor. No consistent or marked changes occurred in the relative concentrations of the various fatty

TABLE 1

Effect of in vivo dissimilation of sodium succinate and DL-*aspartic acid on rumen liquor composition and pH*

Trial No.	Treatment	Sampling time	Total VFA	Acetic	Propionic	Butyric	Valeric plus higher acids	Rumen liquor
		(hr.)	*(μM/ml)*	*(Molar % total VFA)*				*(pH)*
1	Previously fasted 18 hr.	0	91.4	68.7	16.1	13.2	1.9	6.9
		0.5	87.8	68.7	16.0	13.4	1.8	7.0
		2	78.2	68.5	16.1	13.3	2.1	6.9
		5	75.2	68.0	15.9	13.3	2.8	6.8
		8	54.8	68.5	15.3	13.6	2.6	7.0
2	1 kg. sodium succinate	0	21.3	69.5	18.9	11.6[a]	....	....
		0.5	49.6	66.5	19.7	13.8	....	....
		1.5	55.1	65.3	22.3	12.4	....	....
		3.5	56.4	61.5	27.6	10.9	....	....
		5.5	66.6	53.9	35.7	10.4	....	....
		8	47.1	57.4	32.4	10.2	....	....
3	1 kg. DL-aspartic acid (unneutralized)	0	47.9	68.2	15.0	12.8	4.0	7.5
		0.5	35.8	69.7	15.1	9.0	6.2	4.5
		2	24.1	67.3	15.2	8.1	9.4	5.4
		5	18.1	66.2	17.9	9.4	6.5	6.5
4	1 kg. DL-aspartic acid (unneutralized)	0	66.5	66.0	15.7	13.0	4.4	7.6
		0.5	50.6	68.2	15.8	11.7	4.3	5.7
		2	39.2	67.6	17.2	13.4	1.8	6.5
		5	43.5	65.0	19.8	11.6	3.6	7.0
5	1 kg. DL-aspartic acid (neutralized to pH 6.9 with Na_2CO_3)	0	70.7	66.8	15.3	15.1	2.8	6.5
		0.5	60.3	67.0	15.3	14.2	3.5	7.1
		2	58.4	65.1	18.3	13.5	3.1	7.2
		5	64.2	62.7	22.5	10.9	3.9	7.2
		8	69.4	62.4	24.4	9.7	3.7	7.3

[a] Includes valeric and other higher acids.

acids. However, deamination did occur. This is in agreement with the findings of Sirotnak *et al. (18)*. Lactic acid was not observed in these rumen liquor samples.

Glucose. The D-glucose dissimilation data are shown (Table 2). The principal in vivo changes were increases in rumen propionic, butyric, and lactic acid concentrations, and decreases in rumen liquor pH and free ammonia concentration. Valeric acid concentration did not undergo any appreciable changes. A large decrease in free ammonia concentration of the rumen liquor adds confirmation to the view that ammonia utilization is enhanced by the presence of readily available carbohydrate. Increases in propionic acid concentration also were observed during the dissimilation of glucose by WCS. The presence of large amounts of glucose in the rumen appeared to affect the activity of the bacteria, because the WCS prepared 5 hr. after administering glucose attacked this substrate more vigorously and with a greater production of propionic acid than did the WCS without previous exposure to this substrate.

Maltose. In vivo maltose dissimilation (Table 3) did not result in appreciable changes in the relative proportions of VFA. Total VFA concentration increased rather markedly in most cases. This is made evident by comparing the maltose data with those of the control cow (shown in Table 1). During these trials, a relatively large increase in lactic acid occurred which, when combined with the increase in total VFA, resulted in a marked decrease in rumen pH. It will be noted that the lactic acid concentration following the administration of maltose was much greater than that from glucose in Trial 6 (Table 2). This observation agrees with the results of Robinson *et al. (15)*.

The results of the in vitro dissimilations of maltose by cell suspensions were similar to the in vivo results. The proportion of acetic acid was lower, and that of propionic and butyric acids higher, than in the case of the in vivo trials. For proper evaluation, the butyric plus higher acid fraction in the dissimilations by cell suspensions should be compared with the combined butyric and valeric acid fractions of the in vivo trials. The one major exception noted was the large increase in the propionic acid concentration of the 0-hr. WCS dissimilation with added maltose (Trial 12).

DL-*lysine.* The in vivo and in vitro data (Table 4) indicate that little or no VFA were produced from DL-lysine. Sirotnak *et al. (17)*, using L-lysine, obtained similar results. It will be noted that there was a decrease in total VFA in the rumen (absorption) and an increase in rumen pH. Only slight increases were noted in rumen lactic acid concentrations.

Glycine plus DL-*alanine.* The in vivo data provided little or no evidence of VFA production from the mixture of glycine and DL-alanine, although there was a large production of ammonia (deamination) and some production of lactic acid. The in vivo and in vitro dissimilations of the glycine and DL-alanine mixture were in close agreement, in that both indicated that the mixture, if fermented at all by the rumen microorganisms, produced negligible amounts of VFA. The uniform pH of the rumen liquor during these trials served as supporting evidenec for the preceding statement. These observations verify the results of Sirotnak *et al. (17)*.

TABLE 2

In vivo and in vitro dissimilations of glucose by rumen microorganisms

Trial No.	Treatment	Sampling time	Total VFA	Acetic	Propionic	Butyric	Valeric plus higher acids	Rumen liquor	Rumen liquor lactic acid	Rumen liquor free ammonia
		(hr.)	*(μM/ml)*		*(Molar % total VFA)*			*(pH)*	*(μM/ml)*	
6	1.5 kg. glucose added in vivo	0	57.2	68.5	13.0	14.3	4.2	7.1	0.0	7.2
		0.5	50.0	63.1	16.0	18.1	2.8	6.6	2.5	4.3
		2	73.5	57.6	21.5	18.9	2.0	6.3	3.2	4.5
		5	69.5	57.9	21.5	18.5	2.1	6.9	0.0	1.0
7	1.5 kg. glucose added in vivo	0	101.5	67.8	16.6	11.9	3.7	6.6	....	....
		0.5	89.5	61.8	19.3	16.1	2.8	6.0	....	....
		2	105.0	57.6	19.4	21.2	1.8	6.0	....	....
		5	93.0	56.3	19.3	21.8	2.6	6.5	....	....
8[a]	In vitro WCS from Trial 6	0-hr.	15.0	68.0	13.5	9.1	9.4	Control, no substrate		
		0-hr.	19.5	65.0	16.8	10.4	7.3	400 μM glucose added		
		5-hr.	12.6	58.5	20.1	14.1	7.3	Control, no substrate		
		5-hr.	35.5	60.9	28.0	8.2	2.9	400 μM glucose added		

[a] Dissimilations by WCS prepared from rumen liquor obtained before (0-hr.) and after (5-hr.) adding glucose in Trial 6 above.

TABLE 3

In vivo and in vitro dissimilations of maltose by rumen microorganisms

Trial No.	Treatment	Sampling time	Total VFA	Acetic	Propionic	Butyric	Valeric plus higher acids	Rumen liquor	Rumen liquor lactic acid
		(hr.)	*(μM/ml)*	*(Molar % total VFA)*				*(pH)*	*(μM/ml)*
9	1.5 kg. maltose added in vivo	0	101.3	64.2	17.5	11.3	7.0	6.6	0.0
		0.25	85.0	61.8	18.4	14.1	5.7	6.1	11.0
		0.5	91.5	63.1	19.3	13.2	4.4	6.0	13.0
		2	140.2	62.0	18.1	16.1	3.8	5.6	4.5
		5	125.8	62.7	16.9	16.4	4.0	5.9	0.45
10	1.5 kg. maltose added in vivo	0	114.4	62.4	16.3	15.2	6.1	6.7	0.0
		0.25	100.8	60.6	19.0	14.8	5.6	6.2	8.7
		0.5	111.9	62.1	17.6	14.8	5.5	6.0	11.6
		2	129.4	59.3	18.4	17.6	4.7	5.8	5.1
		5	134.8	59.4	15.5	18.8	6.3	6.0	0.11
11	1.5 kg. maltose added in vivo	0	122.1	59.9	20.8	14.2	5.1	6.0	.....
		0.25	121.5	61.4	20.7	13.6	4.3	5.6	.....
		0.5	127.4	58.5	19.8	13.2	8.5	5.5	.....
		2	120.6	59.1	20.4	15.7	4.8	5.2	.....
		5	97.3	60.7	18.9	15.3	5.1	4.9	.....
12[a]	In vitro WCS from Trial 9	0-hr.	29.1	55.7	18.7	25.6[c]	Control, no substrate		
		0-hr.	46.2	52.9	28.2	18.9	200 *μM* maltose added		
		5-hr.	34.1	52.0	24.9	23.1	200 *μM* maltose added		
13[b]	In vitro WCS from Tria 10	0-hr.	21.1	53.5	23.0	23.5[c]	Control, no substrate		
		0-hr.	30.5	55.2	22.6	22.2	200 *μM* maltose added		
		5-hr.	34.0	53.5	23.0	23.5	200 *μM* maltose added		

[a] Dissimilations by WCS prepared from rumen liquor obtained before (0-hr.) and after (5-hr.) adding maltose in Trial 9 above.
[b] Dissimilations by WCS prepared from rumen liquor obtained before (0-hr.) and after (5-hr.) adding maltose in Trial 10 above.
[c] Includes valeric and other higher acids.

TABLE 4

In vivo and in vitro dissimilations of DL-*lysine by rumen microorganisms*

Trial No.	Treatment	Sampling time	Total VFA	Acetic	Propionic	Butyric	Valeric plus higher acids	Rumen liquor	Rumen liquor lactic acid
		(hr.)	*(μM/ml)*	*(Molar % total VFA)*				*(pH)*	*(μM/ml)*
14	1.5 kg. 4 -lysine added in vivo	0	50.4	69.6	21.4	8.5	0.5	7.4	0.0
		0.5	54.2	71.1	18.1	10.5	0.3	7.0	0.18
		2	49.9	73.4	17.8	8.5	0.3	7.2	0.0
		5	42.5	78.6	18.9	1.7	0.8	7.4	0.0
15	1.5 kg. DL-lysine added in vivo	0	38.0	64.2	19.5	13.0	3.3	6.9	0.0
		0.5	35.5	65.4	18.3	11.6	4.7	6.8	0.33
		2	33.8	66.0	18.1	12.6	3.3	7.1	0.0
		5	33.5	65.0	17.8	14.7	2.5	7.0	0.0
16[a]	In vitro WCS from Trial 17	0-hr.	17.0	68.3	22.3	9.4[b]	Control, no substrate		
		0-hr.	18.0	69.3	16.6	14.1	200 *μM* DL-lysine added		
		5-hr.	17.0	66.8	17.6	15.6	Control, no substrate		
		5-hr.	17.0	73.6	16.3	10.1	200 *μM* DL-lysine added		

[a] Dissimilations by WCS prepared from rumen liquor obtained before (0-hr.) and after (5-hr.) adding DL-lysine in Trial 14 above.
[b] Includes valeric and other higher acids.

DISCUSSION

In three trials, the in vivo and in vitro results were in close agreement qualitatively. Quantitative differences existing between in vivo and in vitro results could be owing to intraconversions among the VFA and to absorption occurring during the in vivo trials. The results of the in vivo fermentation of succinate was similar to the in vitro results reported by Doetsch *et al. (4)*. The failure to obtain any apparent fermentation of aspartic acid in vivo is attributed to the abnormal pH of the rumen contents caused by the DL-aspartic acid. The extremely high acidity (approximately pH 4.5) may have adversely affected rumen bacterial metabolism and interfered with normal dissimilation. When aspartic acid was partially neutralized to pH 6.9 with Na_2CO_3, it was attacked by rumen microorganisms and resulted in a marked increase of VFA, mainly propionic acid. The in vivo results of partially neutralized aspartic acid are similar to in vitro results of WCS reported by Sirotnak *et al. (18)*.

It is of interest to note that in vivo trials with glucose indicated that the presence of fermentable carbohydrates enhances ammonia utilization by rumen microorganisms. It was noted also that the in vivo fermentation of maltose and glucose resulted in the production of lactic acid, as well as VFA. The lactic acid reached a maximum concentration within 2 hr. after the administrations of the substrates and in 5 hr. had almost completely disappeared from the rumen. This high concentration of lactic acid may be explained by assuming that it is a normal intermediate in carbohydrate metabolism in the rumen, and that its formation is occurring at a faster rate than its dissimilation and/or absorption. Similar results concerning lactic acid production have been reported by Waldo and Schultz *(19)*.

DL-lysine or a combination of glycine and DL-alanine was not fermented to VFA by rumen microorganisms. These results agree with in vitro WCS results of Sirotnak *et al. (17)*. However, glycine plus DL-alanine was deaminated in vivo by rumen microorganisms. The identity of the compound(s) resulting is as yet unknown. Loosli *et al. (12)*, Black *et al. (2)*, and Duncan *et al. (6)* have provided evidence demonstrating the synthesis of essential amino acids by rumen microorganisms. Perhaps reactions in the rumen involving essential amino acids are entirely assimilatory in nature. Deamination processes would aid in supplying ammonia required for bacterial (McDonald, *13*), and possibly host, protein synthesis.

Observations made on rumen liquor pH values during in vivo trials indicated that the pH of the rumen liquor is directly related to the total VFA concentration of the ruminal contents. It is thought that these measurements, although not representing the precise intraruminal pH, do reflect any large changes in rumen liquor pH values.

During the in vitro trial with glucose (Trial 8, Table 2), differences were observed between the fermentation of the added glucose by the 0-hr., as compared to the 5-hr., WCS. It should be noted that increases in butyric acid occurred during both the in vivo and in vitro trials. However, with added substrate, the in vitro concentration of butyric acid by the 5-hr. WCS did not keep pace with

the in vivo concentration increases, although a considerable increase did occur. These results indicate that the in vivo administration of relatively large amounts of certain carbohydrates can alter the activity of rumen microorganisms. The effects of certain carbohydrates on lactic acid and polysaccharide production by bovine rumen bacteria have been reported by Robinson *et al. (15)* and Gibbons *et al. (8)*. Alterations may occur for one or more of the following reasons: *(a)* a bacterial population in which one or more species have increased in numbers; *(b)* an increased metabolic activity of one or more species of microorganisms; or *(c)* the establishment of a metabolic shunt pathway in the presence of excess substrate by one or more species of rumen microorganisms.

Although qualitative in vivo and in vitro results were similar, quantitative differences were noted. Variations may be the result of differential absorption of end-products, changes in pH plus other in vivo factors, and altered bacterial metabolism. These differences also could be owing to enzyme inactivation in processing cell suspensions, exclusion of protozoa, omission of important bacteria because of adherence to various material, lack of cofactors for certain reactions, assimilations, and/or utilization of some VFA, owing to the absence of absorption.

The in vitro cell suspension method appears to be a useful technique for studying toxic and/or expensive substrates; for artificially altering the chemical environment in a predictable manner and direction; for pharmacological investigations, and for pilot studies for subsequent animal experiments.

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NUTRITIONAL FACTORS IN CALF DIARRHEA[1, 2]

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SUMMARY

The influence of various components of milk-type diets on consistency of feces has been evaluated. Calves were fed modified milk (whole or skimmilk) diets to determine the effects of fat, lactose, minerals, and curd formation, individually and in all possible combinations. The presence of milk fat (3%) reduced diarrhea. Increasing the mineral content of the milk to four times normal by addition of simulated whey ash produced moderate diarrhea; increasing lactose to twice normal increased diarrhea to a lesser extent. The prevention of curd formation with sodium citrate did not increase the incidence of diarrhea. The response to minerals may involve additional factors since, under slightly different conditions in this study, the mineral content of milk diets was increased to ten times normal without causing diarrhea.

Diarrhea is a primary concern in raising diary calves. Not only is diarrhea a major factor contributing to the high rate of calfhood mortality, but it lowers resistance to other diseases, slows the growth rate, and reduces the efficiency of feed utilization. No doubt, infectious agents are often involved; however, nutritional (and managemental) factors also deserve attention. Phillips *et al. (16)* reported that vitamin A and members of the vitamin B complex were beneficial as preventives of diarrhea in milk-fed calves, but other studies *(11, 15)* failed to substantiate this work. Since the advent of whole-milk replacers, a knowledge of the possible influence of the various constituents of milk and milk products on the diarrheic nature of the diet has become of greater practical and fundamental importance. Wing *et al. (21)* suggested that the diarrhea produced during the feeding of a whole-milk replacer cmoposed primarily of dried when product may have been the result of the high mineral content in this diet and/or of the absence of curd formation in the stomach. However, the specific influence of these factors, or the other major constituents of milk, on calf diarrhea has not been investigated extensively.

The present study was designed to examine further the relation of certain components and characteristics of milk and milk products to the characteristics of calf feces. Two experiments were employed: a preliminary experiment (I), to explore the contribution of lactose, minerals, butter oil, casein, lactalbumin, and curd formation to the diarrheic property of the diet of calves, and a subsequent experiment (II), to evaluate critically the influence of lactose, minerals, milk fat, and curd formation as independent and interacting factors affecting color and consistency of feces.

Received for publication May 29, 1957.

[1] Journal Paper No. J-3218, Iowa Agricultural Experiment Station, Ames. Project No. 1324.

[2] Supported in part by funds provided by Western Condensing Co., Appleton, Wis.

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EXPERIMENTAL PROCEDURES AND RESULTS

Experiment I

Procedure. Preliminary tests were made to obtain a general appraisal of the effects of certain milk-type diets on consistency of feces. Seventeen calves from the Iowa State College dairy herd were used. Details of the design of each succeeding phase of these tests were determined in part by the results of the preceding phase. Calves remained with their dams for three days, then were moved to a calf barn and, in most cases, were placed in individual wooden pens provided with water and wood-shavings bedding. Calves used in the test of curd tension were housed in pens having metal-screen bottoms and metal and wood sides.

The basal diets employed were whole milk, reconstituted skimmilk,[4] and semisynthetic diets, supplemented during most of the experiment with 100 mg. of a commercial trace-mineral mixture, 500 U.S.P. units of vitamin D_2 in irradiated yeast, and 50 mg. of chlortetracycline as Aurofac D[5] per calf daily. All supplements were stirred into the milk (about 37° C.) immediately before each feeding. Diets were administered via nipple-pail twice daily. The diets (Table 1)

[4] Spray-process nonfat dry milk solids.

[5] Supplied by Fine Chemicals Division, American Cyanamid Co., Pearl River, N. Y.

TABLE 1

Influence of modified and semisynthetic milk diets on fecal consistency

(Experiment I)

Supplementation of basal diet	Basal diets				
	Whole milk	10% Dried skimmilk (reconstituted)	2.4% Lactalbumin suspension	2.4% Casein suspension	14% Whey product suspension
	Rate of feeding *(% body weight)*				
	8	10	8	8	10
	Average scours index[a]				
None		0.2(2)[b]			2.4(9)
Minerals I (MI), 2.1%[c, d]	1.0(7)				
Minerals II (MII), 2.1%[d]	1.0(5)	0.6(2)			
Lactose (L), 5.0%[d]	0.6(7)	1.3(11)			
Butter oil (BO), 3.0%					1.8(2)
KOH, to neutralize					2.4(5)
L + MI[e]	0.2(8)				
L + MII	0.8(18)	2.0(16)	0.8(10)	1.2(4)	
L + BO		0.9(10)			
BO, 2.8% + lecithin, 0.2%					1.7(6)
BO + MII + L		0.6(8)	0.7(5)	0.9(3)	
L + MII + lactalbumin, 1.0%	0.7(5)				
L + BO, 2.8% + lecithin, 0.2%		0.2(3)			

[a] Scours index = fecal-consistency evaluations on the scale 0 to 4, denoting normal feces through watery diarrhea. Observations included in the average began 24 hr. after diet administration and continued to a maximum of six observations (made twice daily) per trial.

[b] Numbers in parentheses represent number of trials in which each diet was fed.

[c] Higher levels, up to 6.3%, were used in several trials.

[d] Amounts added provided a total daily intake of these nutrients equal to or slightly greater than the amount in the whey-product diet.

[e] Amounts used in the last seven diets are the same as shown above, unless otherwise indicated.

were formulated to imitate, in various respects, the composition of a reconstituted whey product[6] which was found to be rather consistently laxative. The apparent inconsistency in levels of feeding of diets (shown in Table 1) was the result of an attempt to equalize certain of the nutrients and, at the same time, provide energy sufficient for maintenance requirements, at least. When lactose and minerals were added to the milk, the resulting levels of intake of these nutrients were equal to or slightly above the levels resulting when whey product was fed.

The mineral levels in reconstituted whey product (2.04%), compared to the amount in whole milk (0.7%), prompted trials in which whole milk was supplemented with a mineral mixture (Minerals I)[7] containing the principal elements of milk ash. Minerals I subsequently was replaced by Minerals II,[8] which more closely simulated whey ash. During several of the first trials, mineral-supplement levels were increased gradually up to 6.3% of the milk fed. The butter oil was homogenized into the diet at 2,000-lb. pressure. Potassium hydroxide was added to certain whey diets to increase the pH from about 4.7 to 7.0. The curd tests were made on Holstein milk; portions of the high- and low-curd milks were separated soon after the afternoon milking. Duration of the trials usually was between two and six days, but in some instances extended to periods of over 2 wk.

Observations on the color and consistency of feces were made twice daily (A.M. and P.M.) by manual stimulation of defecation. Body weights and temperatures also were obtained daily.

Results. The average consistency of feces of calves on the various diets is shown (Tables 1 and 2). Minerals added to whole milk at levels of from one to nine times the amount normally present in milk apparently had no serious effect on fecal consistency. Except for a few occasions, at the highest levels of minerals, these diets were readily consumed. Only two calves of the 12 on high-mineral diets showed appreciable diarrhea, and continued feeding of these diets for

[6] Dried whey products was furnished by Western Condensing Co., Appleton, Wis. Its proximate percentage composition was: nitrogen-free extract, 57.6 (lactose, 46); protein, 17.2; ash, 14.6; fat, 0.4, and fiber, 0.0.

[7] Minerals I contained 300 g. $Ca_3(PO_4)_2$, 300 g. KCl, and 400 g. NaCl.

[8] Minerals II contained 356 g. $CaHPO_4$ (added as an equivalent amount, 450 g., of $CaHPO_4 \cdot 2H_2O$), 360 g. K_2HPO_4, 175 g. $MgSO_4$, and 390 g. NaCl.

TABLE 2

Effect of curd tension (CT) on the laxativeness of milk diets

(Experiment I)

Supplementation of basal diet	Basal diets[a] *(10% of body weight)*				
	Whole milk			Raw skimmilk	
	Noncurd	Low CT	High CT	Low CT	High CT
	Average scours index[b]				
(None)	0.4(3)			0.3(2)	
Lactose, 5% + minerals, 2.1%		1.7(3)	1.6(6)	2.1(5)	1.7(4)

[a] Noncurd milk (from one cow) did not form curd in vitro. Low CT milk averaged about 30 g. of curd tension. High CT milk averaged about 60 g. of curd tension.

[b] Scours index = fecal-consistency evaluation made twice daily on the scale 0 to 4, denoting normal feces through watery diarrhea.

periods of over 2 wk. was generally accompanied by firm feces. After prolonged feeding of whole milk, containing abnormally high amounts of minerals, most of the calves exhibited long, rough coats, unthriftiness, and occasional tremors. Lactose added at 5% of the whole-milk diets, with or without mineral supplementation, seemed to have no influence on consistency of feces; similarly, lactalbumin had no consistent effect.

With reconstituted skimmilk as the basal diet, lactose supplementation produced moderate looseness, and lactose plus mineral supplements resulted in very loose feces. The latter was true also when lactose and minerals were administered in either high- or low-curd tension milk (Table 2). Inclusion of butter oil, with or without lecithin, in the high-lactose or high-lactose-plus-mineral diets markedly reduced the diarrheic effects.

Feeding reconstituted whey product consistently produced a greenish-black, watery diarrhea, even when the pH was raised to approximately 7 before feeding. Diarrhea was even more severe than that observed after the feeding either of skimmilk or of lactalbumin suspension supplemented with lactose and minerals. Butter oil, with or without lecithin, homogenized into the whey-product diet, tended to delay the appearance of the diarrhea several days. None of the semisynthetic diets containing lactalbumin or casein was consistently diarrheic.

Chewing on the pens and eating the wood-shavings bedding were common after calves were from 5 to 6 wk. of age. Though calves changed little in body weight during this study, body temperatures and general appearance indicated that no serious disease conditions were involved.

No noticeable difference in consistency of feces was evidenced from feeding calves milk of different curd tensions (Table 2). Moderate diarrhea resulted when raw skimmilk and whole milk were supplemented with lactose and minerals at the two levels of curd tension tested. Calves used in this study acquired chronic looseness of feces which resisted return to normalcy; consequently, some of the anticipated tests were not made. The apparent discrepancy between the results presented in Table 1 and those in Table 2, for feeding a whole-milk diet supplemented with lactose and minerals, appears to be related to the type of pens (and bedding) used. The aforementioned group may have received an antidiarrheic effect from consuming some of the shavings used for bedding (see Discussion).

Experiment II

Procedure. The design of this experiment was based primarily upon the results from preliminary studies. A factorial trial was employed, using an 8×8 quasi-Latin square *(3),* in order to test more critically some of the factors explored during preliminary work.

Four factors (fat, lactose, minerals, and curd formation) were tested separately and in all possible combinations, requiring a total of 16 diets. Eight Holstein male calves were assigned at random to ration sequences (also established randomly) composed of eight diets. This permitted the testing of each diet by four calves and a total of 64 individual trials.

Diets were tested during four-day periods, with minimal three-day adjustment periods of whole-milk feeding allowed before subsequent test feedings. When severe diarrhea occurred, the scheduled feedings were discontinued. Scheduled dietary sequences were continued as soon as feces maintained normal firmness for two days.

At four days of age, calves were removed from their dams and placed in new, individual calf pens. These pens were fitted with expanded-metal-screen bottoms and had metal partitions and tile walls, minimizing the consumption of extraneous material by the animals. A basal whole-milk diet was fed for from four to 11 days of age at 8% of body weight, in the manner used in the preliminary experiment. Experimental feeding was begun at 11 days of age, at 8% of body weight (adjusted weekly for weight increases). Trace minerals[9] and a vitamin-antibiotic mixture[10] were added to all diets after four days of age.

The milk fed was a mixture composited after the afternoon milking of equal amounts from five Holstein cows, and contained about 3.1% fat. A portion of this was separated for the skimmilk diets (testing about 0.02% fat). Amounts needed for morning feedings were stored overnight in a refrigerator. For the test of fat on fecal consistency, the results of diets with whole milk were compared to those containing skimmilk. The amounts of lactose (5%) and minerals (2.1%) used were selected because of the effects observed in preliminary studies (see Table 1). For the test of curd formation, 3 ml. of a 60% solution of sodium citrate (dihydrate) was added per pound of milk. In tests with young fistulated calves, this procedure prevented curd formation, as determined by palpation of the abomasal contents through the rumen wall. The effectiveness of sodium citrate also was verified in diets containing milk with or without lactose and minerals at levels used in this experiment.

Consistency and color of feces were noted twice daily by observing the feces on the concrete floor beneath the floor grating. Body temperatures were recorded daily and weights weekly.

Results. The mean fecal-consistency evaluations for the four calves fed each diet are presented (Table 3). It is noted that the three most diarrheic diets contained both lactose and minerals and had an average effect of moderate laxation. Feces usually were dark green in color, sometimes almost black. A greenish effect was especially common during mineral supplementation. In most instances, calves were normal in fecal consistency at 11 days of age, when experimental feeding was scheduled to begin, and usually recovered from any laxation of preceding trials during the three-day recuperation period.

Since the distribution of the scours-index values was approximately Poisson, the data were transformed as follows:

$$\sqrt{\text{Scours index} + 0.4}\,.$$

[9] Eight ml. added to the milk of each calf per feeding of a mineral solution composed of $MgSO_4 \cdot 7H_2O$, 1,355.0 g.; $FeC_6H_5O_7 \cdot H_2O$, 133.0 g.; $MnSO_4 \cdot H_2O$, 33.8 g.; $CuSO_4 \cdot 5H_2O$, 8.8 g.; KI, 1.5 g., and $Co(C_2H_3O_2)_2 \cdot 4H_2O$, 1.0 g., made up to 4,000 ml. with distilled water.

[10] One capsule per calf daily, containing 2.3 g. of the following mixture: chlortetracycline HCl (crystalline), 25 g.; irradiated yeast (9,000 USP units/g), 8.3 g., and vitamin A palmitate feed supplement (5,000 USP units/g), 250 g.

TABLE 3

Mean fecal-consistency observations for calves fed modified milk diets
(Experiment II)

Supplementation of basal diet	Basal diets[a]	
	Whole milk	Skimmilk
	Average scours index[b]	
(None)	0.3	0.7
Minerals II,[c] 2.1%	0.8	1.2
Lactose, 5.0%	1.0	0.6
Sodium citrate, dihydrate, 0.40%	0.3	0.8
Citrate+ Lactose[d]	0.3	0.8
Citrate + Minerals II	0.6	1.4
Lactose + Minerals II	2.0	1.7
Citrate + Lactose + Minerals II	1.0	1.9

[a] Fed at 8% of body weight.

[b] Average of fecal-consistency evaluations made twice daily on the scale 0 to 4, denoting normal feces through water diarrhea. Observations included in the average began 24 hr. after diet administration and continued to a maximum of six observations per trial.

[c] Composed of mixture of 356 g. $CaHPO_4$ (added as its equivalent, 450 g., $CaHPO_4 \cdot 2H_2O$); 360 g. K_2HPO_4; 175 g. $MgSO_4$, and 390 g. NaCl.

[d] Rate of supplementation is the same in combinations as when added singly.

The analysis of variance of the transformed data indicates significant influences of milk fat and lactose at $P = 0.05$ and of minerals at $P = 0.01$. Table 4 gives the mean main effects and interactions both for the original and for the transformed data. Minerals and lactose, respectively, were found to have a positive effect toward producing laxation; whereas, butterfat had a negative, or antidiarrheic, action. These effects were independent of the effects of other factors. The absence of curd formation in the abomasum, as produced by the action of sodium citrate, did not appear to influence consistency of feces.

TABLE 4

Estimate of effects of nutritional factors on fecal consistency

Factor	Mean effects	
	Scours index[a]	$\sqrt{\text{Scours index} + 0.4}$[b]
Fat (F)	—0.401	—0.177[c]
Curd-formation (C)	—0.214	—0.094
Lactose (L)	0.338	0.137[c]
Minerals (M)	0.808	0.335[d]
F × C	—0.245	0.103
F × L	—0.224	0.083
F × M	—0.141	—0.014
C × L	—0.130	—0.042
C × M	—0.078	—0.030
L × M	0.172	0.075
F × C × L	—0.104	—0.084
F × C × M	—0.069	0.047
F × L × M	0.250	0.049
C × L × M	0.264	0.102

[a] Scours index = fecal-consistency values based on the scale 0 to 4, denoting normal feces through watery diarrhea.

[b] Standard error of the mean main effects and two-factor interactions = 0.066, and of the three-factor interactions = 0.076.

[c] Significant at $P = 0.05$.

[d] Significant at $P = 0.01$.

Feces were voided by normal calves at approximately 24- to 36-hr. intervals; in very young calves much longer intervals sometimes were observed. Weight changes during the course of this experiment were small; however, calves appeared to be vigorous at all times.

DISCUSSION

Milk fat was the only factor investigated which significantly reduced the laxativeness of milk diets. Studies of Wing *et al. (21)* suggested this role of fat in calf diets, an effect which had been observed earlier with white rats fed high melting-point triglycerides *(20)*. Gullickson *et al. (9)* found that calves fed diets containing butter oil were less affected by scours than those fed diets containing oils having lower melting points. The cause for the costive action of fat has not been determined.

It seems possible that the effect of fat in reducing the diarrhea associated with high levels of minerals in the diet may be related to *(a)* the effect of fat in increasing mineral utilization *(2, 12)*, *(b)* conjugation of the fatty acids liberated from the dietary fat with the dietary minerals, reducing osmotic pressure by a decrease in molecular entities, *(c)* complexing of bile acids with fatty acids, and *(d)* providing a protective coating to the mucosa, reducing sensitivity of the intestinal tract.

The suggestion of a diarrheic effect from lactose, observed both in the preliminary and in the final study, agrees with previous reports *(1, 8, 17)*. Increased acid production in the intestinal tract has been suggested *(6, 14)* as the cause of the digestive disturbance. Should this be correct, the rapidity of the onset of diarrhea from high-lactose diets suggests a correspondingly rapid conversion of bacterial flora to aciduric types. A hydragogue effect also has been suggested as the mode of action of lactose in increasing intestinal motility *(7)*.

A high level of minerals seems to be a definite factor in the cause of much of the diarrhea which occurred during these studies. Reducing the mineral content of whey by dialysis prevented diarrhea in rats *(5)*, and adding the dialysate back to the dialyzed whey restored its laxative property. Though no direct evidence is available to explain the phenomenon of mineral laxation, the following seem to be possibilities: *(a)* Inorganic ions may have a direct hydragogue effect which is considered the mechanism of certain mineral laxatives, such as magnesium sulphate. The salts of magnesium and the sulphates and phosphates of sodium and potassium are effective in producing increased fluid retention in the intestine through osmosis *(18)*. Distention of the tract by the large liquid content provokes contraction and increases passage of tract contents. *(b)* Minerals may stimulate indirectly the secretion and/or excretion of bile, which is recognized as possessing laxative action *(18)*. The greenish appearance of feces characteristically observed during the feeding of high levels of minerals may be the result of the excretion of large amounts of bile *(19)*. The presence in the feces of this pigment might be the result of an abnormally large amount of excreted bile or of the incomplete transformation of biliverdin to stercobilin,

the principal pigment of normal feces. It has been shown that magnesium sulphate, a constituent of the mineral supplement used in these experiments, promotes evacuation of bile from the gall bladder by dilation of the bladder sphincter *(10)*. It seems possible, then, that the role of fat in reducing, or preventing in some cases, mineral laxation may be owing to the complexing of fatty acids with the bile acids in the formation of choleic acids *(10)* and/or other compounds. The bile thereby might lose its laxative effect. *(c)* Minerals also might inhibit lactose hydrolysis and galactose utilization and indirectly induce intestinal hypermotility. Such an effect has been claimed for calcium chloride *(4)*. *(d)* Since fatty acids may activate the passage of secretin into the blood stream *(10)*, complexing of minerals with fatty acids possibly might impair this process and disturb pancreatic digestion.

The absence of an adverse effect from feeding curd-inhibited diets is contrary to the results of others *(1, 13)*. Blaxter and Wood *(1)* state that when clot formation is inhibited, scours inevitably follow. However, the absolute effect of coagulation was not ascertained in their work, since the diets used either were abnormally low in casein or contained gelatin; thus, an adequate control diet (containing like ingredients) was not employed.

The contrasting effect of added lactose and minerals in whole milk, fed to calves in different type pens, might be attributable to the wood-shavings bedding used in the earlier studies, as no other aspect of these trials differed greatly. A costive effect may have been produced by tannin in the shavings eaten by the calves housed in pens bedded with shavings. Tannic acid tends to arrest diarrhea by allaying intestinal inflammation through an astringent action *(18)*. Whether the amount of tannin present in the shavings was sufficient to have a significant effect is not known. It is also possible that the effect of shavings was partially or entirely physical in nature. The shavings may have absorbed moisture or, merely by mixing with the residue from digestion, may have altered the consistency of feces. It must be recognized also that although bedding appeared to be the only major difference to which the variations in diarrhea (other than those owing to diet) could be attributed, other possible differences of environmental and genetic nature can not be ignored completely.

Since the feeding of a lactalbumin suspension containing lactose and minerals to simulate whey product did not demonstrate any consistent laxative effect, it might be speculated that the composition of the whey product used in this study differed in some of the following respects from the simulated whey-product diet: *(a)* A lactose-lactalbumin complex may be present as a result of the browning reaction occurring during the heat treatment in processing of some whey. *(b)* The ratio of α- to β-lactose may differ, in the whey product employed, from that in the commercial lactose used. *(c)* Other components present in relatively small amounts in the whey product may be of significance. The laxative effect of whey product, then, seems to be owing to factors other than its protein and appears to be partially, but not entirely, attributable to its high mineral content. Lactose also may be involved, but probably to a lesser extent. The absence of

curd-forming potential and low pH appear unrelated to the laxativeness of the whey-product diets.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Mr. Lantis Ratcliff in management of the calves.

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FERTILITY RATES OF COWS BRED FOLLOWING ESTROUS CYCLES OF VARYING LENGTHS[1, 2]

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SUMMARY

This study shows no practical reason for delaying service because of estrous cycle irregularity, if the cow is otherwise normal and the cycle is at least 17 days. The conception rates for cycles of less than 17 days were 34% ($P < 0.01$) among normal cows not bred at the previous estrus, as compared with 50% for all services in this grouping. Similarly, those bred following intervals of 26-27 days were generally below average, but were not significant. Conception rates among otherwise normal cows which failed to conceive at the previous estrus were significantly lower for cycles of 20–22, 32–34, and 35–37 days, and were significantly higher for cycles over 50 days in length. The average was 40% for all services in this cow-group. Among otherwise abnormal cows not bred at the previous estrus, conception rates were significantly lower for estrous intervals of 2–16 (19%), 26–28 (21%), and 29–31 (12%) days in length. This cow-group averaged 29%. Only cycles occurring 50–100 days after a previous breeding had significantly higher conception rates in the group of abnormal cows (26 *vs.* 21%). Abnormal cows repeating for service anytime within 37 days profitably might have been withheld from service (19–22 days, a possible exception), because the chances for conception were one-third greater for cycles exceeding 43 days in length. The study involved 19,369 services following estrous cycles of known lengths. The data were from one herd of Holsteins for a 25-yr. period.

It is frequently recommended that cows not be serviced following estrous cycles of irregular length. This generally means cycles occurring outside an interval of 18–25 days. Since over 40% of all estrous cycles can be expected at intervals shorter or longer than 18–25 days, a large number of cows becomes involved *(5)*. The question arises as to the effect on the chances for conception when cows are serviced at the termination of an irregular cycle. Ellenberger and Lohman *(3)* observed from 274 cycles that conception rates were 40, 55, and 48%, respectively, for cycles shorter than 17, 17–25, and over 25 days. Moeller and VanDemark *(7)*, using 4,885 repeat-service cycles from artificial breeding, observed 180-day nonreturn rates of 34, 49, 45, 50, and 54%, respectively, for cycles of 2–17, 18–25, 26–35, 36–50, and 51–72 days after the previous insemination. Only the 2–17 day group differed significantly from the mean of all. Flerchinger and Erb *(6)* and Olds and Seath *(9)* have made similar observations.

From previous work *(3, 6, 7, 9)*, it was not possible to determine what effect genital abnormalities and previous service had on conception rates. An added bias in artificial breeding data is that cows with long returns to service are more likely to be sold without rebreeding and thus appear as a nonreturn. This would

Received for publication November 13, 1957.

[1] Scientific Paper No. 1673, Washington Agricultural Experiment Stations, Pullman. Project 888.

[2] This work was suuported in part by funds from Regional Project W-2, "Causes and Prevention of Breeding Failure in Dairy Cattle." W-2 is a cooperative study by agricultural experiment stations of the Western Region and the Dairy Research Branch, USDA.

tend to make nonreturns for repeat services higher for intervals over 40 days. Some evidence for this is suggested from the data of Moeller and VanDemark *(7)* and Flerchinger and Erb *(6)*.

The present study was undertaken to compare conception rates between nonservice and repeat-service cycles of variable lengths, for normal and abnormal cows.

MATERIALS AND METHODS

Breeding records from the Carnation Milk Farms' Holstein-Friesian herd at Carnation, Washington, were used. These records were supervised by a single herdsman and extended from 1926 to 1950. Resident veterinarians recorded results of clinical examinations on individual cow-history cards. On the opposite side of the card the herdsman recorded estrus, service sire, and calving dates. Methods of housing and general management were quite constant during the experimental period. Conditions were such that all cows could be observed readily during the day, whether on pasture, in box stalls, or in exercise pens. Although observation at night was not ideal, some cows in the herd were milked four times daily throughout this period and all were milked at least three times daily. This reduced the time cows were not under observation. Natural service was used before 1940, and artificial service with semen containing no antibiotics was used exclusively after 1940. Erb *et al. (5),* in a previous study of these data, found no significant differences between natural and artificial breeding. Therefore, the two periods were not considered separately in this study.

Morrison and Erb *(8)* previously have reported the reproductive capacities and sterility rates among cows in this study.

The data were coded on International Business Machine cards by date of occurrence for estrus, service, service sire, reproductive abnormality, calving date, and type of birth. A separate card was used for each event occurring throughout the lifetime of the cows. By creating a time sequence covering the experimental period, it was possible numerically to identify each date and to calculate the intervals electronically by simple subtraction.

Estrous cycles were classified according to (1) normal, (2) abnormal, (3) service, and (4) nonservice. A cow which had no reproductive abnormality observed during the reproductive period (after calving to the next calving or until disposal) was termed normal. Normalcy of estrous cycles or normalcy of calving was not involved when characterizing cows as normal. Such calving abnormalities as twinning, abortions, still-births, and retained placentae were found to have no significant influence on estrous cycle distributions, provided the reproductive tract was normal subsequently *(5)*. Only those abnormal calvings which were associated with later reproductive abnormalities were in the abnormal group of reproductive periods. Therefore, the characterization of normal cows was based on the absence of gross clinical symptoms, not involving calving or length of the estrous cycles, during the reproductive period. Undoubtedly, some cows characterized as normal were not, because of relatively minor and unobserved cases of infection in the reproductive tract.

Estrous cycles which commenced with breeding at the previous estrus were classified as service (repeat-service) cycles. Those when service was not involved at the previous estrus were classified as nonservice cycles. This classification was considered important, to eliminate partially the effects of early embryonic mortality *(11)* from one group of cycles. The influence of service sire was not completely removed from the nonservice cycle group, because occasionally a cow was not bred at the next estrus subsequent to breeding. The next estrus in these instances was classified as a nonservice cycle.

The conception rate was determined for each cycle length within type of cycle. As shown (Table 1), these were combined into subclasses in order to have sufficient services upon which to estimate conception rates. The 1 and 5% confidence limits *(10)* were calculated for each subclass mean and compared with the mean observed for each type of cycle grouping (see footnote, Table 1).

RESULTS AND DISCUSSION

There were 19,369 services (Table 1) following cycles of known lengths. The study averaged 50, 40, 29, and 21%, respectively, for normal nonservice, normal service, abnormal nonservice, and abnormal service cycles. These values are somewhat lower than those previously reported *(8)*, because all services to infertile cows are included, and because cows conceiving at the first estrus after calving would not appear in these summaries.

Among breedings following normal nonservice cycles, only the 2–16 day group had a conception rate significantly below the average for all normal nonservice cycles (Table 1). Greater differences were found among the normal service cycles. Normal service cycles 50–100 and over 100 days in length had signifi-

TABLE 1

Conception rates following service at cycles of varying lengths

Days in estrous interval	Normal cows				Abnormal cows			
	Nonservice cycles		Service cycles		Nonservice cycles		Service cycles	
	Services	Conceived	Services	Conceived	Services	Conceived	Services	Conceived
	(No.)	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
2–16	118	34[a]	89	30[b]	157	19[a]	146	16
17–19	326	48	491	35[b]	389	30	595	19
20–22	1,175	50	1,548	36[a]	1,326	30	1,766	21
23–25	571	53	645	39	651	32	892	17
26–28	137	41	192	35	156	21[b]	267	15[a]
29–31	49	53	140	32[b]	74	12[a]	132	15
32–34	48	40	103	23[a]	60	33	128	16
35–37	58	46	149	31[b]	77	31	176	15[b]
38–43	294	52	510	42	346	32	582	23
44–49	242	46	315	42	254	29	400	26[b]
50–100	476	54	787	49[a]	564	27	927	26[a]
Over 100	114	51	201	59[a]	170	24	356	26[b]
Total	3,608	50	5,170	40	4,224	29	6,357	21

[a] Confidence limits outside of group mean at 1% level of probability.
[b] Confidence limits outside of group mean at 5% level of probability.

Subclass confidence limit = mean $\pm$ '"0.01 or 0.05 $\sqrt{\frac{p(1-p)}{n}}$.

cantly higher conception rates, and the 2–16, 17–19, and 20–22 day cycles were significantly below average as compared with all normal service cycles. This compares favorably with earlier observations for repeat service cycles *(6, 7)*.

The 26–34 day normal nonservice cycles as a group averaged 43% ($P>0.10$), with considerable variation between the 26–28, 29–31, and 32–34 day subclasses (shown in Table 1). In comparison, the conception rates for 29–31, 32–34, and 35–37 day normal service cycles were significantly lower than the average for all normal service cycles.

Conception rates for abnormal nonservice cycles were much lower than those for normal nonservice cycles. Although at a lower level the trends were similar. The 2–16, 26–28, and 29–31 day cycles were significantly lower than the average for all abnormal nonservice cycles. Conception rates for abnormal service cycles were generally below the group average, except for intervals exceeding 43 days ($P>0.01$). In this group, the average conception rate was 26% for intervals of 44–49, 50–100, and over 100 days. It is of interest that the conception rates for the longer service intervals (normal and abnormal) were comparable with the conception rates of their respective nonservice cycles, but were considerably lower for all other groups. This suggests that time may be an important factor in correcting temporary infertility in repeat-breeding cows.

Delaying service one estrous cycle among repeat-service cows may be economically important when they repeat for service within 35 days. Further study of this problem is of practical importance. There also may be a basic explanation, because Black *et al. (1, 2)* have observed that repeat-breeding cows have more resistance to infection than first-service heifers and cows. These observations suggest that such uteri do not offer a suitable environment for implantation, or that they interfere with sperm survival during the sperms' passage.

From these data, and the data of others *(3, 6, 7, 9)*, there appears no practical reason for delaying service because of estrous cycle irregularity, provided the cow is otherwise normal. One might consider delaying service following cycles of 16 days or less (normal cows), because little time would be lost in waiting for the next cycle. Such cows may be temporarily cystic and require prompt treatment to avoid permanent development of the condition. Abnormal cows repeating for service anytime within 37 days profitably might be withheld from service (19–22 days, a possible exception), because the chances for conception are over one-third greater for abnormal service cycles exceeding 43 days. These chances may be even greater, because some cows in this study were withheld from repeat service; thus, when again rebred, their data would appear in the nonservice summary.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the generosity of Carnation Milk Farms, Carnation, Washington, for providing records for this study. We are especially indebted to Henry Duve for the thoroughness with which he kept the breeding records over the years; to Russell L. Pfeiffer, Manager of Carnation Milk Farms, for making office records available, and to Dr. Roger A. Morrison for his tabulation of the original data.

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EFFECT OF PULVERIZED LIMESTONE AND DICALCIUM PHOSPHATE ON THE NUTRITIVE VALUE OF DAIRY CATTLE FEED [1]

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SUMMARY

The effect of pulverized limestone and dicalcium phosphate supplements on the nutritive value of dairy cattle feed was studied, with 12 dairy heifers receiving either an all roughage or a mixed ration. Results of these experiments indicate that pulverized limestone when added to both all roughage and mixed rations for dairy heifers decreased the digestibility of both protein and energy. Similar amounts of dicalcium phosphate did not have this effect. The feeding of dicalcium phosphate along with pulverized limestone decreased the depressing effects of the limestone. These results would indicate that the feeding of calcium in excess of requirements, particularly in wide calcium : phosphorus ratios, may reduce the digestibilities of protein and energy. These results further indicate a need for additional work on the effects of different levels of calcium and phosphorus on feed utilization.

Feedmen and dairy husbandry research workers believe generally that the calcium content of the ration usually is adequate to satisfy the requirements of cattle when legume forage is fed, but that it may be inadequate when most of the forage is nonlegume. They advocate that calcium or calcium-phosphorus supplements such as pulverized limestone, steamed bone meal, defluorinated rock phosphate, or dicalcium phosphate, or a combination of two of these, be added to commercial dairy rations at a rate of 1 or 2% of the grain mixture.

Some researchers oppose calcium supplementation of dairy feeds. These workers say that contrary to common beliefs and practices, cows at average to moderately high levels of milk production, fed liberal allowances of fair-quality roughages and enough concentrate mixture to balance the ration, do not require added calcium supplements. In their opinion, the practice of forcing cattle to eat calcium supplement with all their grain not only is unnecessary and uneconomical but also undesirable from the standpoint of good nutrition. This idea is contrary to the general belief that mineral supplementation, while it may be costly, never harms the animal or affects the use of the ration, when fed within the limits of tolerance.

Relatively little work appears to have been done to determine if supplemental calcium does affect the nutritive value of the ration adversely. Møllgaard *et al. (3)* in 1941 found that the digestion coefficient of organic matter in A.I.V. silage was lowered when calcium carbonate was added to the ration. The decrease was as high as 15.2%. These results were obtained in metabolism experiments with cows. Axelson *et al (1)* from two digestion trials in 1949 with wethers, concluded that there was no beneficial effect owing to the addition of calcium carbonate.

Received for publication April 15, 1957.

[1] Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 205.

The authors became interested in this problem when they failed to increase the digestibility of grass silage by neutralizing the acid with pulzerized limestone. Digestibility of both protein and energy were decreased, rather than increased. The results with silage posed the important question of the harm which addition of limestone to the ration might do. This study was undertaken to obtain quantitative information on the effects of supplemental calcium on the digestion and utilization of protein and energy by dairy cattle.

EXPERIMENTAL PROCEDURE

The experiments to determine the effect of pulverized limestone and dicalcium phosphate on the nutritive value of dairy cattle feed were carried out during the winter and spring periods between 1950–51 and 1954–55. Twelve dairy heifers between 18 and 24 mo. of age were used as experimental subjects.

In the 1950–51 season, six heifers were used in three sets of exploratory digestion experiments, using Ladino clover–bromegrass silage preserved with molasses, timothy silage preserved with sulfur dioxide, and timothy silage preserved with molasses. Silage made up the entire ration, with the exception that half of the animals fed each silage received 100 g. of pulverized limestone per day. All animals were given a cobalt supplement and allowed free access to plain salt (NaCl) bricks.

During the 1951–52 season, a more comprehensive series of experiments was carried out with a grass-legume silage preserved with molasses. Two levels of limestone were fed in addition to the basal ration. The plan of this work is given (Table 1). The animals were given supplementary cobalt as well as free access to plain salt (NaCl) bricks.

TABLE 1

Plan of experiments with grass-legume silage preserved with molasses (1951–52)

Animal No.	Period 1	Period 2	Period 3
11	Silage	Silage plus 50 g. limestone	Silage plus 100 g. limestone
17	Silage plus 50 g. limestone	Silage plus 100 g. limestone	Silage
18	Silage plus 100 g. limestone	Silage	Silage plus 50 g. limestone

In the 1954–55 experiments,[2] the rations fed consisted of mixed grass hay and a 16% crude protein concentrate mixture, 50-50 by weight. In this experiment, both pulverized limestone and dicalcium phosphate were fed as indicated in the plan of the experiment (Table 2). The mineral supplementation is given as a percentage of the concentrate portion of the ration. In this experiment, the animals were allowed free access to trace-mineralized bricks.

The procedure and methods generally used in the laboratory were followed. These consist of a 10- to 14-day preliminary period, during which the animal

[2] The Shea Chemical Co., Adams, Massachusetts, furnished the dicalcium phosphate and pulverized limestone used in the 1954–55 experiments.

TABLE 2

Plan of experiments with mixed ration

(1954–55)

Animal No.	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
G4	2% limestone	2% limestone + 2% dicalcium phosphate	2% dicalcium phosphate	0-minerals	1% limestone	1% dicalcium phosphate
G5	2% limestone + 2% dicalcium phosphate	2% dicalcium phosphate	0-minerals	2% limestone	1% limestone	1% dicalcium phosphate
H35	2% dicalcium phosphate	0-minerals	2% limestone	2% limestone + 2% dicalcium phosphate	1% dicalcium phosphate	1% limestone
H36	0-minerals	2% limestone	2% limestone + 2% dicalcium phosphate	2% dicalcium phosphate	1% dicalcium phosphate	1% limestone

becomes adjusted to the kind and amount of the ration being evaluated. A collection period of 10–14 days follows. During this period, the feed eaten and the separately collected urine and feces excreted are carefully weighed, sampled, and analyzed. At the end of the collection period, each animal then is put in the respiration chamber for two consecutive 12-hr. periods, during which the heat and methane production are measured.

Although the methods of chemical and caloric analyses are those conventionally used, and given in the latest edition of A.O.A.C., some changes have been made in the sampling and in the method of combustion of the silages and feces. The protein analyses of the excreta, as well as the silages, were made on the fresh material. The heat of combustion of the silages and feces was determined on the fresh material according to the method given by Colovos *et al. (2),* using an adiabatic calorimeter. However, the urine was dried in a vacuum oven at 60° C. before it was burned.

RESULTS AND DISCUSSION

The protein and energy content of the silages, hay, and grain fed during the experiments is shown (Table 3).

TABLE 3

Composition of silages, hay, and grain as fed

Feed	Moisture	Protein	Gross energy
	(%)		(Cal/g)
		1950–51	
Ladino-brome molasses silage	76.30	3.164	1.0372
Timothy-SO_2 silage	64.68	2.610	1.8019
Timothy-molasses silage	65.32	2.559	1.7599
		1951–52	
Mixed grass-legume-molasses silage	72.65	3.894	1.3374
		1954–55	
Hay	8.37	6.387	4.4251
Grain	9.73	16.819	4.4637

The effect of pulverized limestone on the digestibilities of the protein and the energy of the grass silages, used in the experiments of 1950–51 and 1951–52, are shown (Tables 4 and 5). When 100 g. of pulverized limestone were added to the all-silage ration of the dairy heifers, it caused a depression in the digestibility of the protein and the energy in all three silages fed to the animals during the 1950–51 experiments.

When the experiment was repeated in 1951–52 with three dairy heifers, with each animal being on a 0-, 50-, and 100-g. pulverized limestone daily intake, along with grass silage as the sole ration, the results of the previous year were confirmed. The addition of 100 g. of limestone again caused a significant depression in the digestibility of both protein and energy. There was not a significant effect in the 50-g. limestone intake.

The effects of limestone and dicalcium phosphate, fed in graded amounts alone and in combination, on the digestibility of the protein and the energy

TABLE 4

Effect of limestone on the digestibility of the protein and the energy of silages

(1950–51)

	No limestone[a]			100 g. Limestone/day[a]		
	Animal No.	Protein	Energy	Animal No.	Protein	Energy
		(%)			(%)	
Ladino-brome silage with molasses	16	57.52	60.64	11	53.32	53.81
	12	52.32	51.92	13	51.38	49.86
	15	55.72	57.73	14	52.92	52.48
	Av.	55.25	56.76	Av.	52.54	52.05
Timothy-SO_2 silage	12	48.19	58.37	11	46.55	57.03
	15	49.42	59.86	14	46.12	56.05
	Av.	48.80	59.11	Av.	46.33	56.54
Timothy-molasses silage	16	42.49	58.52	11	40.89	57.50
	15	40.37	55.60	14	39.39	54.95
	Av.	41.33	57.06	Av.	40.14	56.22
Over-all averages		48.46	57.64		46.34	54.94

[a] The differences between 0 and 100 g. limestone per day for both protein and energy were significant at the 1% level.

TABLE 5

Effect of limestone on the digestibilities of the protein and the energy of grass-legume silage preserved with molasses

(1951–52)

	No limestone[a]		50 g. limestone/day		100 g. limestone/day	
Animal No.	Energy	Protein	Energy	Protein	Energy	Protein
			(%)			
11	65.01	62.04	63.73	57.87	63.38	60.09
17	63.73	64.11	64.97	62.40	61.88	58.48
18	64.80	62.30	65.14	65.10	64.16	58.49
Av.	64.51	62.82	64.61	61.79	63.14	59.02

[a] Differences between 0 and 100 g. limestone for both protein and energy are significant at the 5% level.

of a mixed ration in the experiments of 1954–55, are shown (Table 6). Results of those experiments show that the 2% limestone decreased the digestibility of both protein and energy, thus confirming the previous work with silage. The 2% dicalcium phosphate did not have any appreciable effect. The addition of 2% dicalcium phosphate to the 2% limestone supplement minimized the depressing effect of the limestone. The 1% limestone supplement decreased the digestibility of the energy significantly, but its depressing effect on the protein of the ration was not significant. The 1% dicalcium phosphate did not affect the digestibility of the energy or the protein of the ration.

The data suggest that the addition of calcium to the ration depressed the digestibilities of both protein and energy; whereas, the addition of phosphorus increased the digestibility of the protein. The change in the calcium : phosphorus ratio does not explain entirely the results obtained, particularly with the mixed

TABLE 6

Effect of limestone and dicalcium phosphate on the digestibilities of the protein and the energy of a mixed dairy ration
(1954–55)

Animal No.	0-Minerals		2% limestone[a]		2% dicalcium phosphate[b]		2% limestone + 2% dicalcium phosphate		1% limestone[c]		1% dicalcium phosphate[d]	
	Energy	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy	Protein
	(%)											
G 4	57.60	65.56	53.61	62.04	59.77	65.86	58.15	64.96	57.04	64.19	60.36	63.52
G 5	63.03	66.26	60.67	64.65	58.67	67.76	59.35	65.70	59.40	65.10	60.95	69.08
H 35	61.58	66.01	56.16	64.94	60.30	67.62	61.45	65.71	57.93	67.63	58.86	70.13
H 36	58.94	67.91	53.95	65.11	61.44	67.01	60.28	65.44	58.35	67.73	60.57	71.66
Av.	60.29	66.43	56.10	64.18	60.04	67.06	59.81	65.45	58.18	66.16	60.18	68.60

[a] Differences between 0 and 2% limestone are significant for both protein and energy.
[b] Differences between 0 and 2% dicalcium phosphate are not significant.
[c] Differences between 0 and 1% limestone in energy are significant at the 1% level, but the differences in the protein are not significant.
[d] Differences between 0 and 1% dicalcium phosphate for both energy and protein are not significant.

rations. It appears that the amounts of calcium and phosphorus also are factors. When the 2% limestone was added to the mixed ration, the digestibilities were depressed considerably. The addition of both 1 and 2% dicalcium phosphate tended to increase the digestibility of protein. When increased quantities of both calcium and phosphorus were added, the digestibility values were between these two extremes. There is nothing in these data which would incriminate the CO_3 radical for the decrease of digestibility, but this possibility should not be ignored. These results suggest the need for studies on the effect of calcium and phosphorus supplements, fed separately and in various ratios, on the digestibilities of protein and energy, as well as on feed consumption and growth.

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VALUE OF AN ORGANIC ARSENICAL IN THE RATION OF YOUNG DAIRY CALVES [1]

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SUMMARY

This study reports the results of supplementing the daily ration of the young dairy calf with 60 and 120 mg. of sodium arsanilate from the third day through the 8th wk. of age. Neither growth rate and efficiency of feed utilization, nor incidence of scouring, was significantly influenced by the presence of the supplement in the ration. No symptoms of arsenic toxicity were observed in any of the animals. These data indicate *(1)* that no beneficial effects can be expected from supplementing the ration of the young, healthy calf with sodium arsanilate and, *(2)* that the young calf is tolerant to high levels of this organic arsenical.

Inorganic arsenic has been used therapeutically in animals for well over 100 yr. *(7)*. The use of organic arsenicals in animal feeds has been reviewed by Frost *et al. (8)* and by Frost and Spruth *(9)*. Some evidence has accumulated which indicates that certain of the organic arsenicals have growth-promoting and/or disease-controlling properties for the young calf.

Graf and Holdaway *(11)* observed that calves fed arsanilic acid scoured less and made more rapid gains during the first 4 wk. of life than did animals not receiving the supplement. Slightly better results were obtained at levels of 60 and 120 g. per ton than when a 240-g. level was fed. Dewey *(5)* did not obtain any response from dairy calves supplemented with sodium arsanilate at levels of 20 or 40 mg. per 100 lb. body weight. However, some indication was obtained in the Texas work *(5)* that unthrifty calves might benefit from relatively small doses of sodium arsanilate or dodecylamine *p*-chlorophenylarsonate. In a study of the comparative value of several antibiotics and 3-nitro-4-hydroxy phenyl arsonic acid for young calves, Owen *et al. (17)* found that the arsenical gave results equal to or better than the antibiotics, in maintaining an increased rate of gain after the supplements were removed from the animals' rations.

Bartley *et al. (1)* supplemented the diets of calves from birth to 23 wk. of age with 50 mg. of arsanilic acid per calf daily. No statistically significant difference in weight gain or feed efficiency was observed between the supplemented and the control calves. Also, there was no apparent difference between the two groups in incidence of disease.

In an experiment designed to study the value of supplementing the calf's ration with an arsenical (arsanilic acid) plus an antibiotic (aureomycin) *(12)*, evidence was obtained which indicated that such a combination (60 mg. arsanilic acid and 25 mg. aureomycin per calf daily) was no more effective in increasing weight gains and decreasing the incidence of scours than either of the supplements fed alone at the same levels. The supplements, fed singly or in combination, did

Received for publication December 27, 1957.

[1] Supported in part through funds provided by Abbott Laboratories, North Chicago, Ill.

increase daily gains; however, the differences between the supplemented and the control calves were not significant, because of the large within-group variation. The animals used in this experiment were obtained from various state institutional herds in Virginia.

This study was undertaken *(a)* to obtain additional information on the value of an organic arsenical (sodium arsanilate) in the ration of the young calf and *(b)* to obtain information on the tolerance level of this arsenical for young dairy calves.

EXPERIMENTAL PROCEDURE

Twenty-four Holstein calves (12 males, 12 females) were used in this study. The calves remained with their dams for at least 24 hr. postpartum and subsequently were removed to individual pens and hand-fed colostrum twice daily for an additional 24-hr. period. On the third day postpartum, the calves were placed on the experiment and allotted at random to the following treatments: Group A (control)—basal; Group B—basal plus 60 mg. sodium arsanilate per calf daily, and Group C—basal plus 120 mg. sodium arsanilate per calf daily. The sex distribution was the same in each group.

The experimental treatments were imposed through the 8th wk. of age, during which time each calf received approximately 300 lb. of whole milk. All calves were fed a grain ration composed of the following poundages: ground corn, 600; ground oats, 580; wheat bran, 500; soybean oil meal, 280; iodized salt, 20, and steamed bonemeal, 20. Grain consumption was limited to a maximum of 4 lb. per day. Fair-quality mixed hay averaging 11% total protein was provided ad libitum.

The arsenical was dissolved in water and added to the milk at feeding time. Intake of the supplement approximated 0.007 to 0.013% of the total dry matter in the ration.

Feed consumption data were obtained on a daily basis for all calves. The calves were weighed and measured at the beginning and at the end of the experiment, also at weekly intervals between.

RESULTS

A summary of the growth data is presented (Table 1). The control calves and those receiving 120 mg. of arsenical daily made essentially the same body weight gains, whereas the 60 mg.-per-day group averaged somewhat lower. However, analysis of variance revealed that none of these differences in weight gain was great enough to be statistically significant.

Average increases in height of withers and in chest circumference were very similar in all groups. No significant differences with respect to these body measurements were observed between treatment groups.

The feed data (Table 2) suggest that both levels of the arsenical increased the consumption of hay, whereas the higher level also increased slightly the intake of concentrate. These differences in feed consumption were not significant, however. Statistical analysis revealed no significant differences in efficiency of feed utilization (calculated TDN per lb. weight gain) between treatment groups.

TABLE 1

Effect of arsenical supplementation on weight gains and certain body measurements
(eight calves per group)

Treatment groups[a]	Av. initial weight	Av. daily gain	Av. increase in	
			Wither height	Chest circumference
	(lb.)		*(in.)*	
A	89	0.94 ± 0.05[b]	2.7 ± 0.25	4.4 ± 0.35
B	87	0.87 ± 0.04	2.5 ± 0.24	4.4 ± 0.29
C	86	0.98 ± 0.02	2.5 ± 0.28	5.2 ± 0.34

[a] A = control, B = 60 mg. sodium arsanilate daily, and C = 120 mg. sodium arsanilate daily.
[b] Mean ± standard error.

TABLE 2

Effect of arsenical supplementation on feed consumption and efficiency of feed utilization
(eight calves per group)

Treatment[a]	Av. total consumption per calf				TDN per lb. gain
	Milk	Grain	Hay	TDN[b]	
			(lb.)		
A	305	57.1[c] ± 3.7	11.8 ± 1.6	96.0 ± 3.4	1.84 ±0.63
B	305	54.1 ± 2.6	14.1 ± 2.2	95.8 ± 1.8	2.03 ±0.44
C	305	62.1 ± 0.78	15.1 ± 0.60	101.9 ± 2.0	1.90 ±0.64

[a] A = control, B = 60 mg. sodium arsanilate daily, and C = 120 mg. sodium arsanilate daily.
[b] Calculated from Morrison's *(16)* tables.
[c] Mean ± standard error.

The incidence of scouring was very low in this experiment and was unrelated to treatment.

No clinical symptoms of arsenic toxicity were observed in any of the calves.

DISCUSSION

The data from this study do not support the preliminary findings of Graf and Holdaway *(11)* or the results of Owen *et al. (17)*; however, they do confirm the observations of Dewey *(5)*, as well as the data of Bartley *et al. (1)*. They are also in agreement with the more extensive studies of Graf *(10)*. The reason (or reasons) for these conflicting results is not apparent, but may be associated with the level and types of bacterial infection in the experimental animals. The form of the arsenical used also may be important. On the basis of the present investigation and the growth studies made by Dewey *(5)* sodium arsanilate, at the levels tested, would appear to be of little or no value in the feeding of calves free of any disease of major consequence. The work of Graf *(10)* and Bartley *et al. (1)* indicates the same to be true with respect to arsanilic acid. On the other hand, 3-nitro-4-hydroxy phenyl arsonic acid was reported to exert a clear-cut beneficial effect in the ration of young calves in the Iowa work *(17)*. Further work appears to be needed to establish the possible role and value of arsenicals in calf rations.

One of the major advantages found for the arsenical by the Iowa workers *(17)* was its apparent superiority in maintaining an increased growth rate subsequent to the removal of the supplement from the ration. The present study does not provide any information on this point; however, earlier observations *(13)* do not support this finding.

It might be theorized that the arsenical was ineffective in the present investigation, because diarrhea was not a problem even though the animals were housed in dirty quarters. It is of interest that in the study of Owen *et al. (17)* scouring was quite prevalent, and since none of the supplements fed was effective in controlling the diarrhea, it was proposed to be of noninfectious origin.

The fact that the arsonic acids are important as growth promotants and disease-control agents for swine and poultry *(8)* and of questionable value for young calves and older ruminants *(2, 3)* indicates that the rumen microorganisms might be implicated. With respect to the young calf, this explanation does not seem feasible because, in most of the studies reported, the animals used were essentially monogastric animals during the greater part of the experimental period when the supplement was fed. Also, in light of the recent work by Hegland *et al. (15)*, it seems doubtful that any large amount of the arsenical would enter the rumen when administered in the milk, especially if nipple-pails were used.

The results of the present study, as well as earlier work *(11, 12)*, indicate that both arsanilic acid and sodium arsanilate are tolerated at high levels in calves. In a limited investigation *(14)*, it was found that arsanilic acid fed with the grain ration was slightly toxic at the 0.04 to 0.05% level. There appears to be a rather wide margin of safety for certain of the arsenicals in fattening lambs *(4)* and beef cattle *(2, 6)*.

ACKNOWLEDGMENT

The authors wish to express their appreciation to D. V. Frost, Abbott Laboratories, North Chicago, Illinois, for the arsenicals.

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EFFECT OF ZEOLITE TREATMENT ON HARD DRINKING WATER ON MILK PRODUCTION[1]

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SUMMARY

During November 1 to April 30 (181 days), periods of alternate years, 19 cows averaged 17.6 lb. of milk daily when receiving untreated well-water averaging 190 p.p.m. of total hardness, as compared to 19.9 lb. when receiving water from the same source but zeolite-treated to zero-hardness. The average stages of lactation (after calving) were 169 and 132 days, respectively. When adjusted for stage of lactation by covariance analysis, the corrected yields were 18.8 and 19.0 lb. for untreated and treated water, respectively. This difference is not significant. It is concluded that softening the drinking-water for dairy cows has no measurable effect on milk production. When given a choice between untreated and treated water during an entire summer, the herd showed no clear-cut preference for either, although they drank more of the untreated water.

Water is the nutrient required in greatest quantity by dairy cows, and it plays a critical part in their nutrition. The water for many dairy herds is supplied by deep wells and contains considerable amounts of minerals, with calcium and magnesium compounds usually the most prominent. Henry *(3)* compared rain-water with hard well-water for pigs, with the thought that the calcium of the hard water might be beneficial. However, there was no evidence of any effect, favorable or unfavorable. During recent years, there has been some interest in the possible value of softening the drinking-water of dairy cattle. Graf and Holdaway *(2)* found no difference in milk production or gain in weight due to zeolite treatment of well-water of about 290 p.p.m. total hardness, when supplied to milking cows during 57-day periods. Water consumption, and ratio of milk produced to water consumed, were not affected by treatment of the water. Blosser and Soni *(1)* carried out a similar experiment with 27 cows over 6-wk. periods, with no significant difference in milk production, water consumption, or water : milk ratio. The work reported in this paper was planned to test the effect over longer periods of time.

EXPERIMENTAL PROCEDURE

The entire milking herd of 24 Guernsey cows at the LaCrosse station of the University of Wisconsin was divided into two groups, balanced as nearly as possible as to age and stage of lactation. The water bowls of half of the stalls were connected to a line from a deep well, supplying water averaging 190 p.p.m. total hardness. The remaining bowls were connected to a line supplying water from

Received for publication May 7, 1957.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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the same source, but treated by a zeolite process to zero-hardness. Both lines were metered, to give an accurate measure of total water consumption. The meters were read daily. The softening units were of a tank-replacement type, changed regularly to insure water of zero-hardness at all times.

Daily milk weights were recorded, and fat was determined on monthly samples.

The experiment on the cows was started on November 1 and continued through April 30. During the first winter season, Group A received the zeolite-softened water, and Group B the untreated. During the following pasture season, all cows had access to tanks containing softened water. For the second barn-feeding period the two groups were reversed, Group A getting the untreated and Group B the zeolite-treated water.

The results for the two 6-mo. periods are shown (Table 1).

TABLE 1

Relation of hardness of water to milk production

	Hard water			Soft water		
Cow No.	Days milked	Av. days fresh	Milk *(lb.)*	Days milked	Av. days fresh	Milk *(lb.)*
	Group A			Group A		
112	110	254	896	136	203	1,387
170	149	75	3,259	142	71	3,313
268	181	330	1,598	105	170	2,652
248	124	220	1,032	158	227	1,627
271	181	184	2,198	182	176	3,071
262	132	168	2,184	134	122	2,842
306	130	205	3,122	141	92	2,845
266	177	89	3,689	182	91	3,512
276	107	172	2,302	113	57	2,454
288	119	158	2,616	60	30	1,515
	Group B			Group B		
105	181	95	3,984	182	113	3,792
277	181	163	4,437	182	102	4,310
241	181	270	1,317	128	246	2,654
215	181	125	4,244	182	120	3,679
272	181	184	3,092	182	176	3,532
307	100	75	2,399	137	157	2,455
263	144	72	4,131	140	78	4,543
285	130	144	1,843	123	67	2,222
165	123	232	1,261	94	206	1,419
Av.	148.0	169.2	(daily) 17.6[a]	142.3	131.8	(daily) 19.9[b]

[a] Adjusted for stage of lactation, (daily) 18.8.
[b] Adjusted for stage of lactation, (daily) 19.0.

Because all the milking cows of the herd were used, and the experiment covered a 2-yr. period, it was inevitable that there should be some change in the make-up of the herd. Nineteen cows were in the herd through the entire experiment, and seven were not. These seven are omitted from Table 1, which includes the data on all cows which had a full period on each treatment.

The reversal design of the experiment equalized age of cow between treatments, and to a large extent the differences between the two seasons. However, since the records cover a definite calendar span, the days in milk and the stage of lactation were not equal.

DISCUSSION

Nineteen cows were in the herd during both periods of the experiment. Ten had treated water during the first period, and nine during the second. Each group received the untreated well-water during the alternate periods. These 19 cows averaged 19.9 lb. of milk per day during 142 days in milk on treated water, as compared to 17.6 lb. for 148 days in milk on hard water. During the soft-water periods, the average stage of lactation (average days after calving) was 132 days, as compared to 169 on hard water. The apparent average daily milk production advantage for the treated-water periods was 2.3 lb. However, there was an advantage of 37 days for earlier average stage of lactation.

In an individual lactation, a period of 181 days averaging 132 days post-partum would include the span of from 42 to 222 days, inclusive; whereas, an average stage of lactation of 169 days would take in the span of 79–259 days, inclusive. Since the 79- to 222-day portion is common to both, the earlier stage of lactation would enhance production to the extent that the 42–78 days' yield exceeds the 223- to 259-day production. A comparison of corresponding spans within lactations of these cows during the course of the experiment gave an advantage of 2.8 lb. in average daily milk yield for the earlier period. This suggests that the apparent advantage of the treated water may have been entirely owing to the earlier average stage of lactation.

The data were subjected to covariance analysis, to test this observed difference. The average daily milk yields, adjusted by this treatment for stage of lactation, were 19.0 and 18.8 lb. for treated and untreated water, respectively. The difference of 0.2 lb. is not significant. Therefore, it may be concluded that with the type of well-water supplied to the cows in this experiment, averaging about 190 p.p.m. of total hardness, softening by zeolite had no appreciable influence on milk production. These results over 181-day periods are in agreement with those reported by Graf and Holdaway *(2)* for 57-day periods and by Blosser and Soni *(1)* for 6-wk. periods.

Average daily water consumption for 24 cows differed by less than 1 lb. Because the water was metered by groups and some cows, due to unavoidable herd turnover, were not in the experiment through the entire 2-yr. span, it was not possible to arrive at accurate figures for comparing water consumption per pound of milk produced. If we assume that the 19 cows which were compared for milk production drank a pro-rata share of the total water, the consumption was 3.9 lb. of untreated against 3.4 lb. of treated water per pound of milk produced.

In order to check for possible preference, two similar float-equipped tanks were placed in the barn-lot during the second summer of the experiment. One was supplied by the soft-water line, the other by the hard. Both were metered. The entire herd had free choice between the two types of water, with access to no other. During the 103-day period, they drank 11,230 gal. (56% of total) of untreated and 8,810 gal. (44% of total) of treated water. These observations do not indicate any serious discrimination against either.

ACKNOWLEDGMENT

This project was supported, in part, by the Culligan Zeolite Corporation. The authors wish to express their appreciation to Mr. Ray Highberg, local representative of this corporation, who installed and serviced the softening equipment.

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TECHNICAL NOTES

EFFECT OF FEEDING VARIOUS LEVELS OF 2,6-DI-TERTIARYBUTYL-4-METHYL PHENOL TO LACTATING DAIRY COWS [1]

The antioxidant 2,6-di-tertiarybutyl-4-methyl phenol (BHT) has been approved as an additive to poultry rations and also has been approved for human consumption *(1)*. Although a recent report by DeLuca *et al. (2)* indicated that BHT, when fed at 0.01% of ration (90% dry matter basis), was ineffective in reducing the incidence of copper-induced oxidized milk flavor, the possible effectiveness when higher levels of BHT are fed to lactating dairy cows was recognized and the present study was undertaken to explore this possibility.

Two separate trials were conducted, Experiment I from February to March, 1956, with 16 cows (12 Guernseys and four Holsteins) and Experiment II from November to December, 1956, also with 16 cows (eight Guernseys and eight Holsteins). The animals were selected for producing milk which would exhibit oxidized flavor 72 hr. after the addition of 5.0 p.p.m. of Cu^{++}. The cows were randomly assigned in replicates of four animals each, restricted with respect to breed, to one of four levels of BHT—0.00, 0.01, 0.03, or 0.09% of ration (90% dry matter basis). The BHT supplement [2] was premixed with linseed oil meal, so that 4 g. of this premix would be sufficient for 1.0 lb. of ration, and was mixed into the P.M. grain allowance each day of the feeding period.

The experiments consisted of a 1-wk. standardizing period during which only the basal ration, consisting of grass silage, alfalfa hay, and grain, was fed, and a 4-wk. comparison period, during which BHT was included at the indicated levels. The ration allowance was held constant throughout the experiments. Twice weekly during the entire experimental period, milk samples of the P.M. milking were obtained for organoleptic testing. The samples were tested 72 hr. after milking, independently by two judges, without and with the addition of 1.0 and 5.0 p.p.m. of Cu^{++} from copper sulfate. Once weekly, milk weights were recorded and samples of the A.M. and P.M. milking were composited for butterfat analysis. Milk samples obtained at the termination of Experiment I were assayed for BHT content *(3)*.[3] Statistical procedures as outlined by Snedecor *(4)* were employed in the analysis of the data.

No statistically significant differences were observed in the milk production criteria, either between levels of BHT fed or between the BHT-supplemented groups and the negative controls. The following averages for the comparison period, with their standard deviations, were observed for Experiment I and Experiment II, respectively: lb. of milk, 33.0 ± 10.0 and 28.1 ± 12.6; % butterfat, 5.0 ± 0.6 and 4.7 ± 1.5, and lb. 4% FCM, 37.0 ± 12.0 and 29.6 ± 15.1. Whereas covariance, utilizing initial production and/or days in milk, reduced the size of the experimental error, the nonsignificance of the treatment differences was not altered.

Results of the organoleptic tests are presented (Table 1), expressed as per cent samples free from oxidized flavor. No statistically significant reduction in the incidence of copper-induced oxidized flavor was noted for the BHT-supplemented groups *versus* the negative controls, or between levels of BHT fed when 1.0 p.p.m. Cu^{++} had been added to the milk. This may have been owing to the cows' resistance to the induction of oxidized flavor when this lower level of copper was employed. Considering both experiments, there was a slight progressive reduction in the incidence of oxidized flavor occurrence with increasing levels of BHT fed, with complete inhibition when 0.09% BHT had been fed. In the presence of 5.0 p.p.m. Cu^{++}, a significant reduction in oxidized flavor was observed for the group fed 0.09% BHT and a slight, nonsignificant reduction for the feeding of 0.03%. As previously reported *(2)*, the 0.01% level appeared ineffective in reducing the incidence of copper-induced oxidized milk flavor.

Estimates of the minimum level of BHT to feed to prevent copper-induced oxidized milk flavor were based on regressions of per cent samples free from oxidized flavor on level of BHT fed. The estimates were as follows for Experiment I and Experiment II, respectively: 1.0 p.p.m. of Cu^{++}, 0.06% and 0.08% BHT; 5.0 p.p.m. Cu^{++}, 0.19% and 0.18% BHT of ration (90% dry matter basis). These estimates may be too great for application to field conditions, and no data are available for cows whose milk becomes oxidized spontaneously.

Although some interference was evident in

[1] This study was in part supported by grants from the American Cyanamid Co., Chas. M. Cox Co., and the Chas. H. Hood Dairy Foundation. The authors wish to acknowledge the technical assistance of G. R. Farrington and R. M. Prouty.

[2] The BHT supplement, containing approximately 50% BHT and 50% calcium chloride, was supplied through the courtesy of R. F. Elliott, American Cyanamid Co., Pearl River, N. Y.

[3] Mrs. M. Booth of the American Cyanamid Co., Pearl River, N. Y., conducted the BHT analyses of the milk.

TABLE 1

Effect of level of BHT on incidence of oxidized milk flavor

	Per cent of BHT added to ration				Standard deviation of a cow
	0.00	0.01	0.03	0.09	
Samples free from oxidized flavor			*(%)*		
Fresh					
Experiment I					
Standardizing	100.00[a]	93.75	100.00	100.00	6.25
Comparison	98.33[b]	100.00	100.00	100.00	1.67
Experiment II					
Standardizing	100.00	100.00	100.00	100.00	0.00
Comparison	100.00	100.00	100.00	100.00	0.00
1.0 p.p.m. Cu^{++}					
Experiment I					
Standardizing	68.75	68.75	62.50	62.50	35.59
Comparison	95.00	81.66	98.33	100.00	18.73
Experiment II					
Standardizing	75.00	68.75	62.50	62.50	32.87
Comparison	54.69	73.44	93.75	100.00	28.28
5.0 p.p.m. Cu^{++}					
Experiment I					
Standardizing	0.00	6.25	0.00	6.25	7.22
Comparison	16.67	10.00	21.67	54.74	20.13
Experiment II					
Standardizing	0.00	0.00	0.00	0.00	0.00
Comparison	0.00	0.00	7.81	50.00	15.81
BHT fed			*(g/cow/day)*		
Experiment I	0.00	2.0	5.7	17.4	.....
Experiment II	0.00	2.0	6.4	18.1	.....
Concentration of BHT in milk			*(γ/100 ml.)*		
Experiment I	8	18	52	72	36

[a] Values observed before feeding BHT.
[b] Mean of 15 observations during the feeding of BHT.

the BHT assay, as indicated by a positive response with the controls (Table 1), BHT apparently was transferred to the milk in increasing amounts with the level fed.

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EFFECT OF *PARA*-AMINO SALICYLIC ACID ON GROWTH OF MALE HOLSTEIN CALVES [1,2]

It has been suggested that *para*-amino salicylic acid (PASA) would stimulate the growth of calves when included in the diet *(2)*. A feeding trial has been conducted to test further this possibility.

Twelve male Holstein calves were divided into two groups at random. All calves had received colostrum for at least one day before being placed on trial. They were fed limited amounts of whole milk until ten days of age, along with milk replacement which was started at six days of age and continued through 42 days of age. Each calf received a total of 60 lb. of milk and 39.4 lb. of dry milk replacement. Calf starter was fed ad libitum until a maximum of 4 lb. was being consumed by each calf daily. No antibiotic was included either in the milk replacement or in the starter. The roughage used was second-cutting mixed alfalfa-grass hay and was fed ad libitum. The diet fed the experimental group (Group I) was similar to that fed the control group (Group II), differing only in that it contained PASA at the rate of 90 mg/lb of milk replacement and 50 mg/lb of starter. The experimental animals consumed PASA at a rate of approximately 90 mg/100 lb. of body weight per day. Management and environment were the same for both groups.

Results of the trial (Table 1) show that Group II gained more in body weight, height at withers, and chest circumference than did Group I. The differences in favor of Group II were statistically significant at the 1% level of probability for body weight and at the 5% level for height at withers and chest circumference. Group II also required fewer pounds of TDN per pound of gain than did Group I, indicating a decrease in efficiency of utilization of TDN in the experimental group. These results suggest that PASA, when fed at the rate of approximately 90 mg/100 lb. body weight per day and without antibiotics, was not an effective growth stimulant for Holstein calves.

TABLE 1

Growth performances of calves fed para-*amino salicylic acid as compared to controls* [a]

	Group I (Fed PASA)			Group II (Controls)		
	Initial	6 wk.	12 wk.	Initial	6 wk.	12 wk.
Body weight *(lb.)*	101	136	191	104	144	214
Height at withers *(cm.)*	75.0	80.2	84.9	75.2	80.7	86.7
Chest circumference *(in.)*	30.8	33.9	37.6	31.3	34.7	39.0
TDN/lb gain			2.00			1.84

[a] Data represent means of six calves per group.

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[1] Authorized for publication on September 24, 1957, as Paper No. 2200 in the Journal Series of the Pennsylvania Agricultural Experiment Station.
[2] The *para*-amino salicylic acid used in this work was supplied by Chemo Puro Manufacturing Corp., Newark, N. J.

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TOXICITY OF NITRATES AND NITRITES TO DAIRY CATTLE

During the past decade, the use of nitrogen fertilizers in the production of forage crops has become a wide-spread practice in many areas of the tropics. This practice has led to tremendous increases in yields of total digestible nutrients per acre. In tropical areas with 70–80 in. of rainfall annually, the yields of dry forage per acre can be increased from 4–5 tons per year to 25–30 tons per year. This increase in over-all yield is accompanied by a threefold increase in

the per cent crude protein, resulting in an 18- to 20-fold increase in total crude protein per acre. These yields are phenomenal when compared to forage yields obtained in the temperate zones.

The utilization of these high protein grasses has presented problems. These grasses have a relatively high nonprotein nitrogen fraction, including nitrates, which leads to undesirable effects unless certain specified feeding practices are adopted *(2, 5)*.

Recently, very severe losses from toxic agents of unknown origin occurred at two dairy farms in Puerto Rico. One dairyman lost 23 milk cows from his herd of 60; another lost 28 heifers. In both cases the losses occurred within 48 hr. after the first symptoms. These losses were not fully explained and, as both dairies were utilizing high-nitrogen forage, the possibility that nitrates were the causative agent was investigated. This investigation included four feeding trials. This report describes these trials and presents the results.

The grass, *Pennisetum purpureum,* used in these trials was produced on ¼-, ½-, and ¾-acre field plots. A high nitrate content was obtained by the application of 400 lb. per acre of a 10-10-10 fertilizer one week after cutting, followed 4 wk. later by an application of 800, 1,600, or 2,000 lb. of ammonium sulfate per acre. The grass was harvested and fed when 6 to 8 wk. old, depending on the nitrate concentration desired. Each individual ration was prepared daily by weighing both the fresh, finely chopped grass and the concentrate. All animals received the principal nutrients in excess of their requirements *(3)*. Feces and urine samples were collected manually. Nitrate concentrations in all samples were determined by the method of Swann and Adams *(4),* and the methemoglobin levels by the procedure of Evelyn and Mallory *(1)*.

Trial 1. Two pregnant, nonlactating dairy cows, weighing about 950 lb. each, were placed in feeding pens and maintained on a chopped-grass ration that was free of nitrates. No supplements were given. An excess of fresh grass was available in the feed boxes at all times. After 2 wk., the nitrate-free grass was gradually replaced over a period of 1 wk. by a grass that contained 0.3 to 0.4% potassium nitrate (KNO_3) on a fresh weight basis (1.3 to 1.8% dry weight basis). The high-nitrate ration was then fed continuously for 60 days. The daily consumption of one cow varied from 115 to 175 lb., and averaged 135 lb.; that of the other from 125 to 210 lb., averaging 155 lb/day.

Weekly determinations of methemoglobin showed that the levels varied between 2 and 7%, and averaged 5%. Periodic inspections by a veterinarian revealed no symptoms of toxicity. One animal refused to eat on the 34th day but was normal again the next day. Thirty days after the feeding trial terminated one cow gave birth to a normal calf. The other cow gave birth to a normal calf 45 days after the trial ended.

Trial 2. A dairy herd of eight lactating and four nonlactating cows was switched from a nitrate-free pasture roughage to a high-nitrate, chopped roughage (1.8% potassium nitrate on a dry weight basis). The chopped roughage was fed from 12, 3 by 2.5 by 1.5 foot feeding boxes located in a large feeding pen. An excess of chopped grass was maintained in the feeding boxes at all times. Concentrate was fed at milking time in recommended quantities *(3)*. Feeding of the high-nitrate roughage continued 4 wk. The average consumption of roughage was 87 lb/cow/day over the 4-wk. period (336 cow-days).

Blood samples taken at 8 A.M. on the 22nd day of the trial contained normal amounts of methemoglobin, i.e., 3 to 5%. A veterinarian found the herd normal at the end of the trial. Milk production records revealed a slight decline not associated with the high nitrate content of the roughage.

Trial 3. Two yearling bulls, weighing approximately 400 lb. each, were placed on a balanced ration made up of a low-nitrogen roughage and a high-protein concentrate. After 1 wk., the animals were treated for parasites, and the low-nitrogen roughage was replaced with equal amounts of chopped grass which contained from 0.3 to 0.4% potassium nitrate (1.3 to 1.7% dry weight). This ration was fed for 2 wk. and the methemoglobin remained at normal levels. At the end of this 2-wk. period, the high-nitrate forage was replaced with a forage free of nitrates. Chemically pure potassium nitrate was then added to the forage until it contained 1.7% on a dry weight basis. This required 120 g. of potassium nitrate for the daily ration of each animal. After 2 wk., both animals appeared normal and continued to gain weight. The concentration of nitrates and nitrites in the feces and urine showed that less than 4% of the dietary nitrates was being excreted in the form of nitrates and nitrites. The concentration of potassium nitrate in the roughage was then raised from 1.7 to 3.0%. This required 210 g. of potassium nitrate for the daily ration of each animal. The animals were then maintained on this ration for 30 days, and together they consumed almost 30 lb. of potassium nitrate.

During this period, only 1% of the dietary nitrates was recovered unchanged in the feces, and approximately 3% passed unchanged in the urine. Methemoglobin levels ranged from 5 to 8%. At the end of the trial, the animals were examined by a veterinarian and appeared normal.

Trial 4. The two animals in Trial 3 were switched directly to Trial 4. The potassium nitrate (210 g.) in each daily ration was replaced by an equimolecular quantity of potassium nitrite (177 g.). After 1 wk., the methemoglobin levels were 3 and 7%. The quantity of potassium nitrite in each daily ration was raised to 200 g. Two weeks later the animals appeared normal to the examining veterinarian and the

trial was terminated. Methemoglobin levels were 2 and 3% at the end of the trial.

These trials led the authors to the conclusion that the severe losses mentioned previously were not caused by the nitrates in the forage being fed. This conclusion is based on these facts: *(1)* The losses occurred in herds that were obtaining 75% of their requirements from a forage that contained no more than 1.0% potassium nitrate on a dry weight basis. *(2)* Test animals showed no symptoms of nitrate poisoning and continued to gain weight while being fed rations that contained 3.0% potassium nitrate.

ACKNOWLEDGMENT

The authors wish to express their appreciation for the advice and assistance of Dr. Rosendo Cordero, Professor of Veterinary Science, College of Agriculture, University of Puerto Rico, Mayaguez, P. R.

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OUR INDUSTRY TODAY

NEW DEVELOPMENTS IN THE PROPAGATION OF LACTIC CULTURES: CULTURE MEDIA AND BACTERIOPHAGE INHIBITION [1, 2]

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Lactic cultures which do not produce acid uniformly every day present an important problem to the dairy industry. Studies on the causes of slow acid production, or complete failure of a culture to produce acid, indicate that bacteriophage often is responsible. Since bacteriophage has been recognized as an important cause of culture failure, many recommendations have been made to limit its entrance into cultures and to decrease its activity. Culture selection, sanitation programs, culture rotation, and propagation of mother cultures and preparation of intermediate and bulk cultures in a relatively bacteriophage-free area, have been helpful in decreasing culture failures. However, these methods have not entirely eliminated the bacteriophage problem.

A medium which would support good growth of lactic cultures, but which would not permit bacteriophage development would be of value to the dairy industry, provided that the use of such a medium would not constitute adulteration. The addition to a medium such as milk, of some nontoxic substance that would prevent bacteriophage development but permit good growth of the culture organisms, would also have possibilities. In 1949, Shew *(5)* and in 1956, Reiter *(3)* reported that bacteriophage active against the lactic streptococci require calcium for maximum development. Potter and Nelson *(2)* and Doull and Meanwell *(1)* developed media which were deficient in calcium and which supported growth of lactic cultures. These media were used for fundamental studies on bacteriophage. Reiter *(4)* described a medium, based on milk, in which bacteriophage did not multiply because of a lack of calcium, but in which certain lactic cultures grew normally. The medium was designated PRM (phage-resistant medium).

EXPERIMENTS WITH PRM

A supply of PRM was made available for experimental purposes by Fisons Milk Products Ltd., Loughborough, Leicestershire, England. The material was received in dry form and was reconstituted at the rate of 90 g. powder to 910 ml. of distilled water. After reconstitution, it was heated in flowing steam for 1 hr.

The first series of experiments with PRM were designed to determine whether cell lysis would occur in the medium. Two flasks containing skimmilk and two flasks containing PRM were inoculated with 1% lactic culture (LC-4). One flask of skimmilk and one flask of PRM also were inoculated with 1% of a bacteriophage preparation (B-14) active against Culture LC-14 and containing 10^5 particles per milliliter. The flasks were incubated at 70° F. The flask of skimmilk containing culture only, and both flasks of PRM, were coagulated in 16 hr. The flask containing skimmilk, culture, and bacteriophage was not coagulated in 24 hr. Bacteria-free filtrates were prepared from the material in each flask and the filtrate tested for the amount of bacteriophage present. The following results were obtained:

Media	Bacteriophage concentration
	(particles/ml)
Skimmilk + culture	< 1
Skimmilk + culture + bacteriophage	10^9
PRM + culture	< 1
PRM + culture + bacteriophage	< 1

Another trial was conducted in the same manner, except that Lactic Culture LC-3 and Bacteriophage Preparation B-3 were used. The bacteriophage preparation contained 10^6 particles per milliliter. As in the previous trial, the flask containing skimmilk and culture only, and both flasks of PRM, were coagulated in 16 hr. The flask containing skimmilk, culture, and bacteriophage was not coagulated in 24 hr. Bacteria-free filtrates were prepared and filtrates tested for the amount of bacteriophage present. The following results were obtained:

Media	Bacteriophage concentration
	(particles/ml)
Skimmilk + culture	< 1
Skimmilk+ culture + bacteriophage	10^{11}
PRM + culture	< 1
PRM + culture + baeteriophage	< 1

[1] Published with the approval of the Director of the Purdue Agricultural Experiment Station as Journal Series Paper No. 1223.

[2] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by the Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

The data obtained in these, and in additional trials, indicate that bacteriophage does not multiply in PRM. One would assume that some bacteriophage would be detected in a filtrate prepared from a culture grown in PRM and to which many bacteriophage particles were added. However, it is known that bacterial cells will adsorb bacteriophage in the absence of calcium, and that the cells plus the adsorbed bacteriophage would be removed in the preparation of the filtrate.

Experiments also were conducted to determine the ability of PRM to support growth of the organisms in lactic cultures. In these experiments PRM was reconstituted at the 10, 9, and 7.5% levels. The reconstituted material was heated in flowing steam for 1 hr. before use. When cultures regularly propagated in skimmilk (9% solids) were transferred to both skimmilk and PRM, the coagulation times were very nearly the same, regardless of the level at which PRM was reconstituted. However, when transfers were made from PRM to PRM, the coagulation times generally were greater than those of the same cultures transferred from skimmilk to skimmilk, or from PRM to skimmilk.

Six cultures which had been prepagated regularly in skimmilk were propagated in both skimmilk and PRM (9% solids) for three propagations. Then, both sets of cultures were tested for rate of acid production in milk which had been heated for 1 hr. in flowing steam and cooled to 86° F. The milk was inoculated with 1% of each culture, incubated at 86° F. for 6 hr. and the amount of acid determined by titration. The acidities, calculated as per cent lactic acid, were as follows:

Culture No.	Transfer from skimmilk	Transfer from PRM
	(%)	
4	0.62	0.46
5	0.57	0.41
6	0.60	0.36
12	0.50	0.29
14	0.57	0.42
18	0.70	0.44

These results indicate that cultures grown in PRM do not produce acid as rapidly as cultures grown in skimmilk. Additional trials, in which plate counts were used to determine the rate of growth of lactic cultures in skimmilk and PRM, showed that skimmilk was the more favorable growth medium. The plate counts showed variations among cultures with respect to growth in PRM; the medium was more suitable for some cultures than for others. However, in no instance did the growth rate in PRM exceed the growth rate of the same culture in skimmilk under the same conditions.

Several cultures were available which coagulated milk in a normal manner but which contained bacteriophage in rather low concentration. Bacteria-free filtrates from four such cultures showed from 10^3 to 10^4 bacteriophage particles per milliliter. Because some previous experiments had indicated that it is rather difficult to eliminate bacteriophage from such cultures, it was of interest to determine whether cultures could be freed of bacteriophage by propagating them in PRM. Bacteria-free filtrates from the first propagation in PRM showed no bacteriophage in 1 ml. of filtrate from three of the cultures; one culture yielded a filtrate containing about 50 particles per milliliter. Filtrates prepared from the second propagation in PRM showed no bacteriophage in 1 ml. of filtrate from any of the cultures.

The experiments conducted with PRM indicate that:

Bacteriophage active against the lactic streptococci of dairy cultures does not multiply or cause cellular lysis in PRM when the product is reconstituted with distilled water.

A bacteriophage-carrying culture may be freed of bacteriophage by propagation in PRM.

Lactic cultures do not grow in PRM as well as in skimmilk.

Lactic cultures propagated in skimmilk produce acid more rapidly when transferred to milk than cultures propagated in PRM and transferred to milk.

Lactic cultures show variations with respect to their ability to grow in PRM; the medium is more suitable for some cultures than for others.

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UTILIZATION OF WHEY SOLIDS IN FOOD PRODUCTS[1]

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Whey solids have been consumed in many food products during the last few years. To convert this perishable material into a high-quality food requires careful selection of raw milk and the processing and refrigerating of whey and concentrated whey under controlled conditions. Dumping fluid whey into sewage disposal systems is expensive, and it is unlawful in many states to put whey and dairy wastes into streams and rivers.

The cheese industry, in one respect, is no different from other industries. All industries, to prosper in competitive markets, must constantly change their utilization of by-products, as well their ways of producing and marketing them.

Great strides have been made in utilizing whey solids, a by-product of the cheese industry. A large investment in equipment, and a real concern for high quality, are necessary profitably to produce whey solids that will be accepted by the food industry. Centralized cheese-manufacturing operations with modern processing and drying plants have made it possible to make an economical whey product from milk of good quality.

The quality specifications for whey solids of U. S. Extra Grade, effective July 8, 1954, depend upon the initial processing of the product and the nature of the cheese operation. The following steps are involved in processing whey from good-quality milk:

1. The whey is pumped from the cheese vat to a holding tank and then to a centrifugal separator, where the fat is removed as whey cream. U. S. Extra Grade whey solids must contain not more than 1.25% fat.
2. The whey is next pumped to holding tanks which are connected in series, to maintain a constant flow to the evaporators and to enable the operator to test the quality of the whey from different sources.
3. The whey is next preheated to 220° F. and pumped into three- or four-effect evaporators, where it is concentrated in stages. The temperature in the first stage is 200° F., which is decreased to about 140° F. in the last stage. Thus, whey containing 6% solids is condensed to about 40 to 70% solids.
4. The concentrated whey is cooled to about 35° F., pumped into tanks, and transferred to the final drier. To maintain the highest quality standards, this concentrate is hauled not to exceed 40 miles, in stainless tanks, to be dried.
5. It is necessary to stabilize the lactose in the whey concentrate to the monohydrate stage during the drying process, because the anhydrously dried product is very hygroscopic.

The concentrated whey may be dried by tunnel- or shelf-drying, drum- or roller drying, or by spray-drying.

In tunnel-drying, the concentrated whey, containing 70% solids, flows from the vacuum pan into a vat, where it is seeded with a small amount of dried whey from a previous batch and held not more than 24 hr. to allow the lactose to crystallize. Then it is spread on 1/16- to 1/12-mesh screen trays and dried in a tunnel where air circulates at from 140 to 180° F. At the higher temperatures, drying may be complete in from 3 to 4 hr. The dried whey is relatively nonhygroscopic. There are several variations of the tunnel-drying process.

In the drum-drying process, the whey is concentrated to 50% solids and flows to a series of drum driers. The rolls of each unit are 1/16-in. apart and operate at about 300° F. The whey dries in light-brown sheets which are ground and packaged. The resulting product varies in its hygroscopic property. There are several variations of this process.

Spray-drying of concentrated whey is accomplished with equipment used to dry milk. The lactose should be crystallized before drying; otherwise, a very hygroscopic product will result. The whey is preheated to 180° F. for 20 min., then concentrated to 40% solids. This concentrate is sprayed into the drying chamber at 285° F. The finished product is amorphous and somewhat hygroscopic. The whey proteins are soluble. There are also variations of the spray-drying process.

The following figures represent the average composition of fluid whey and whey solids:

	Water	Fat	Lactose	Whey proteins	Minerals
	(%)				
Fluid whey	94.0	Trace	4.5	0.8	0.7
Whey solids	4.0	1.0	71.0	13.0	11.0

Lactose constitutes about 40% of the total solids of milk. It is a reducing sugar of relatively low sweetness. It has a sweetness value of 16, compared to 100 for sucrose. Lactose is more resistant to acids than sucrose; therefore, is not as easily hydrolyzed or inverted. For this rea-

[1] Presented at Research Conference held at Beltsville, Maryland, October 30, 1957, by the Eastern Utilization Research and Development Division, USDA, Philadelphia, Pa.

son, lactose is absorbed from the intestine more slowly than sucrose. The solubility of lactose is much less than sucrose.

The principal proteins in whey are lactalbumin and lactoglobulin. Commercial lactalbumin is a product which is precipitated by heating acidified whey and includes both the lactalbumin and lactoglobulin fractions. The whey proteins are heat-coagulable, depending on the intensity of heating when milk is processed for the various products. The whey proteins are not coagulated by the enzyme, rennin, or by dilute acids; consequently, they are always found in the whey. They have a high nutritional value.

The whey proteins have definite advantages in soups and in sterile creamed soups because they impart emulsion stability, thus greatly retarding the separation of discrete particles and of colloidal phases. In certain types of soups, where the color and acidity are such that regular milk products can not be used, the use of whey solids will not mask the desired color and will produce a smooth, tender body, also.

The baking industry, the largest single potential user of edible-grade dairy solids, has found whey solids to be functionally and economically basic for bakery products. Whey solids can be used as an additional ingredient, as a blend with other dairy ingredients, and as the complete dairy solids in a formula. They are being used for bread, rolls, fermented sweet products, cakes, cookies, crackers, pie crusts, biscuits, doughnuts, icing, and fillings. The loaves containing whey solids showed advantageously, having desirable crust color, color of crumb, softness, resilience, and shelf-life. Owing to the high percentage of lactose, whey solids can be substituted for dextrose and sucrose in many baking formulas.

In baking, owing to the fact that the whey proteins are not readily affected by the low pH of fermented doughs, they will add to the extensibility of doughs during the initial processing period. Because they are heat-coagulable, they will also contribute to structure during baking.

In ice cream, the use of whey solids at a level of 25% of the total serum solids produces a product with improved body, excellent flavor, and texture. The improvement in the body of the ice cream is attributed to the superior emulsifying and water-holding properties of the whey proteins.

In soft-served ice cream, whey solids can replace about 50% of the solids-not-fat. In sherbets, they are used at the 100% level, thus improving flavor, protein stability, and palatability.

In confections such as fudge, caramel, and coatings, whey solids accentuate added flavors and also contribute a rich, creamy flavor. Owing to the low caramelization point of lactose, whey solids will develop a brown color. Lactose caramelizes at 265° F., as compared with 310° F. for dextrose and 325° F. for sucrose. Whey also extends the shelf-life of candies. Lactose retards crystallization, thus controls graining-out. It is recommended that whey solids be added in various replacement levels, in initial tests to determine optimum levels.

In cheese spreads and cheese foods, whey solids are used effectively to provide a more desirable consistency to the product and to carry flavor.

Including whey solids in comminuted meat products and meat loaves of various types results in better color, moisture retention, less shrinkage, and improved palatability. Their use has recently been approved by the Meat Inspection Division of the USDA.

Food manufacturers are interested in whey solids and research is contributing new uses for this important dairy product.

ABSTRACTS OF PAPERS TO BE PRESENTED AT THE FIFTY-THIRD ANNUAL MEETING of the AMERICAN DAIRY SCIENCE ASSOCIATION

North Carolina State College
Raleigh
June 16–19, 1958

MANUFACTURING SECTION

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M1. Density of milk at low temperatures.* P. D. WATSON, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

The densities of a variety of herd milks at low temperatures were determined precisely to provide data to establish volume-weight relationships more accurately than is possible from the literature. Specially designed pycnometers with a capacity of 46 ml., and refrigerated baths controlled to within ±0.02° C., were used. Duplicate determinations were made at two temperatures (0.95 and 4.95, or 0.95 and 9.85° C.) on 105 milk samples from representative sources. The percentage of milk fat in most samples was determined by the Babcock method, and in some of them by the gravimetric method. The percentage of total solids was measured gravimetrically. The percentages of fat, total solids and nonfat solids covered the following ranges, respectively: 3.1 to 6.4; 11.6 to 16.1, and 8. 2 to 10.2. The range of densities at 0.95, 4.95, and 9.85° C. were, respectively: 1.0313 to 1.0387; 1.0326 to 1.0374, and 1.0298 to 1.0369. Tables relating the density to the fat and solids-not-fat will be presented. The results emphasize that density is more closely correlated to the percentage of nonfat solids than to the percentage of fat in the milk.

*This work was financed by the Agricultural Marketing Service.

M2. Interrelation between conductivity, per cent lactose, and freezing point of milk. I. I. PETERS AND R. R. SHRODE, Texas Agricultural and Mechanical College, College Station.

Lactose content, conductivity, and freezing-point depression were determined on 480 fresh, raw milk samples from individual cows. Per cent lactose was determined by the A.O.A.C. method, using a Rudolph (Routine Model 60) double-field polarimeter. Conductivity readings at 37° C. were made with a conductivity bridge (Industrial Instrument Co., Model RC-16B) and accompanying conductivity cell, whereas the freezing point was determined with a Hortvet cryoscope. The correlaton beitween lactose content and freezing-point depression was 0.25; between conductivity and freezing-point depression, −0.33, and between lactose content and conductivity, −0.75. Considering freezing-point depression as the dependent variable, the multiple correlation involving the three variables was 0.33, the partial standard regressions of freezing-point depression on lactose content and conductivity being, respectively, −0.0034 and −0.3343. Thus, these data indicate that nothing is gained by measuring lactose content in addition to conductivity, if the objective is to estimate freezing-point depression, since lactose content is so highly correlated with conductivity.

M3. Influence of vacuum pasteurization upon the freezing points, total solids, and concentration of milk. J. T. LAZAR, JR., AND R. W. HENNINGSON, Clemson Agricultural College, Clemson, S. C.

Milk was pasteurized at 161, 165, 170, and 175° F. and treated at 22, 24, and 26 in. of vacuum in a DeLaval Vacu-Therm pasteurizer, to determine the amount of concentration of product that occurred. Milk was pasteurized also in a HTST pasteurized at these temperatures. The condensates from the vacuum-treated milk were measured and the concentrations of the samples were computed. Concentrations were determined also by comparing the total solids by the Mojonnier method and freezing points by the Hortvet method of the raw and vacuum-treated samples. Practically no concentration occurred in milk samples pasteurized at less than 175° F., unless the vacuum level was 24 in. or more. Concentration occurred at this treatment level and above. The ranges of concentrations were 1.28 to 1.96% for the condensate and 1.64 to 1.94% for the total solids. A rise occurred in the freezing points of samples treated at less than 175° F. and 24 in. of vacuum. Treatments above this level depressed the freezing points. As indicated by the total solids, there was some concentration in the HTST samples. To determine the amount of concentration due directly to vacuum and temperature treatment, the ap-

parent MTST concentration must be subtracted from the total concentration. The maximum concentration (as calculated on this basis) that occurred in the Vacu-Therm was 1.49%.

M4. Sedimentation of whole milk heated in the Mallory small-tube heat exchanger. H. K. Wilson and E. O. Herreid, University of Illinois, Urbana.

The rate of sedimentation of milk solids, principally proteins, is affected by the preheating temperature of the milk before sterilization. Portions of milk from the same batch were heated from 50 to 320, 300, 260, 220, 180, and 140° F. Average sedimentation rates after 66 days were 116, 52.3, 39.7, 44.1, 38.6, and 42.5, respectively, and raw milk was 23.7. Portions of another batch were heated from 140 to 320, 310, 300, and 290° F., the sedimentation rates were 84.9, 68.3, 44.1, and 33.1, and for raw milk, 25.4. All values must be multiplied by 10^{-11}. Nitrogen analyses indicated at sedimentation equilibrium that 20 to 30% of the proteins precipitated at pH 4.5 to 4.7 remained in the supernatant. The rate of protein separation at a force of 9,744 × G was not affected by the temperature of storage, but was affected by the sterilization temperature. The serum protein nitrogen left in the supernatant in milk stored at 100° F. increased with decreased heating. In milk stored at 40° F., the serum protein nitrogen in the supernatant was uniformly lower. The Fisher-Stern ultracentrifuge was used in these studies.

M5. Effect of treatment of raw milk on heat-liberated hydrogen sulfide.* T. Kristoffersen, The Ohio State University, Columbus.

Observations have revealed that the amount of hydrogen sulfide liberated from milk by heat may vary widely, depending on the treatment. Determination of heat-liberated hydrogen sulfide was made by heating milk at 90° C. for 30 min. (Boyd and Gould, *Journal of Dairy Science,* 1957). The concentrations of hydrogen sulfide released from mixed-herd raw milk varied from 220 to 490 γ per liter. To determine the possible cause for these variations, fresh raw milk was incubated at 37° C. for 8 hr. Hydrogen sulfide determinations were made periodically. Results revealed a decrease in heat-liberated hydrogen sulfide during the first 45 min. of incubation, followed by an increase during the next 3 to 5 hr. and a considerable decrease during the remainder of the period. No appreciable bacterial growth occurred during the first 3 to 5 hr. of incubation. Accelerated growth occurred during the second decrease. Another factor affecting release of hydrogen sulfide from milk was low-temperature preheating. Milk was preheated at from 55 to 70° C. for 30 min., or at 63° C. for from 10 to 60 min. With either method, the more severe the preheating treatment the greater the decrease in heat-liberated hydrogen sulfide. The effect of preheating could be overcome by addition of Versene prior to preheating.

* Supported in part by funds from the American Dairy Association and the Ohio Dairy Products Research Fund.

M6. A study of factors which cause variations in the protein-reducing value of fluid milk. J. T. Cardwell and F. H. Herzer, Mississippi State College, State College.

Factors that cause variations in the protein-reducing substances (P.R.S.) of milk were studied with the modified procedure reported at the Southern Division of the American Dairy Science Association in 1957. The P.R.S. value of milk pasteurized by the vat method was greater than that pasteurized by the high-temperature, short-time method. The higher the heat treatment and/or the longer the holding period, the greater was the reducing value. Homogenized milk had a higher reducing value than unhomogenized milk, under the same conditions of heat treatment. The P.R.S. values increased with greater homogenization pressures. No relationship was evidenced between the fat content and the P.R.S. value of the milk. The P.R.S. values of milk from individual Jersey cows averaged greater than the values from either Guernseys or Holsteins. The addition of re-liquefied nonfat dry milk solids to fresh fluid milk increased the P.R.S. values in proportion to the amount of re-liquefied nonfat dry milk added, as determined by the modified procedure. The study was limited to 12% replacement.

M7. Isolation of milk phospholipids and determination of their polyunsaturated fatty acids. L. M. Smith and E. L. Jack, University of California, Davis.

Lipids were extracted from spray-dried buttermilks with equal volumes of hot ethanol, ether, and chloroform. After solvent removal, the lipids were re-extracted with a 50:50:1 ether-pentane-ethanol mixture, dried with sodium sulfate, and filtered. The bulk of the triglycerides was removed by filtration after precipitation of the phospholipids with acetone. Small amounts of phospholipids remaining in the glycerides were absorbed on a silicic acid column and, after elution with methanol, were added to the acetone insoluble material. The phospholipids were further purified by column chromatography. The amounts of polyunsaturated fatty acids were determined from ultraviolet absorption data obtained before and after isomerization. A representative sample of phospholipids (iodine value 31.6, 3.41% P, 3.85% N) contained, in percentages, 1.6 conjugated dienoic, 4.2 monoethenoid, 4.6 linoleic, 2.0 linolenic, 1.2 arachidonic, and 1.7 pentaenoic acids. The percentages of each acid in the total unsaturated fatty acids (with the corresponding percentages for a typical milk fat, given in parentheses) were: 11 (2.5), 27 (87.8), 30 (4.7), 13 (3.2), 8 (0.9), and 11 (0.9), respectively.

M8. Determination of ammonia and the lower volatile amine concentrations in milk by solvent extraction and gas-liquid chromatography.* C. L. HANKINSON AND D. D. COLE, Ohio Agricultural Experiment Station, Wooster, and The Ohio State University, Columbus.

Published methods for the determination of ammonia and lower volatile amines by gas-liquid chromatography employed celite as the immodile phase, undecanol and liquid paraffin as the liquid phase, and dry nitrogen as the eluting gas. Modifications consisting of (1) higher pressure and (2) progressively increasing temperature resulted in good resolution, with quantitative recoveries. Known concentrations of ammonia, N-methyl, N-ethyl, N-propyl, N-butyl, and N-hexyl amines were separated and titrated while the column temperature increased from 40 to 130° C. in 45 min. at a nitrogen pressure of 60 cm. of mercury. Ammonia and the lower volatile amines were extracted from 20 ml. acid milk filtrate, using a modified Roese-Gottlieb procedure. The filtrate was made alkaline and extracted three times, using a total of 150 ml. ethyl alcohol, 600 ml. ethyl ether, and 600 ml. petroleum ether. The combined extracts were acidified with HCl, evaporated to dryness as the hydrochlorides, and dissolved in ethyl ether. The free amines were released by alcoholic NaOH just before adding them to the chromatographic column. Analyses revealed variable concentrations of the lower volatile amines in milk.

*Supported in part by the Hatch Fund (USDA) and by the Ohio Dairy Products Research Fund.

M9. A method for the determination of citric and lactic acids in dairy products. D. R. FRAZEUR, Purdue University, Lafayette, Indiana.

A three-section silicic acid chromatographic column was used with 2 ml. of water and 3 g. of silicic acid in the lower section; 8 ml. of 0.7 N H_2SO_4 and 12 g. of silicic acid in the middle section; and 4 ml. of 0.7 N H_2SO_4, 1 ml. of water, and 12 g. of silicic acid in the upper section. A 3-g. sample was used for the determination. The eluate was a water-saturated mixture of 40% butanol and 60% chloroform. The effluent was removed from the column in 75, 225, and 300-ml. fractions. A colorimetric procedure specific for hydroxy acids was used to determine the lactic acid which appeared in the first fraction. A titration procedure was used to determine the citric acid which appeared in the third fraction. A 97%, or better, recovery was obtained for concentrations of 0.020 to 3 γ of lactic or citric acid per gram of sample. Amino, fatty, and other organic acids present in the sample do not interfere with the determination. This method has been used successfully with ten dairy products. Very little preparation of sample is necessary.

M10. Distribution between the fat phase and the aqueous phase of certain antioxidants added to whole milk. J. MARTINEZ-MATEO, W. L. CHOATE, AND L. F. EDMONDSON, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

During studies of the effect of antioxidants on the keeping qualities of dried whole milk, it was found desirable to obtain information on the phase distribution of these antioxidants when added to whole milk. Recoveries of butylated hydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA) were made on washed and dried butteroil made from fresh whole milk containing from 0.003 to 0.05% antioxidant. It was found that from 70 to 90% of the BHA could be recovered in the butteroil, and less than 5% of the NDGA added appeared in the butteroil and butter serum fractions. Analysis of BHA in cream fractions obtained by siphoning off successive layers from gravity-separated milk indicated that the distribution of this antioxidant between phases was not dependent on the fat globule size. The effects of homogenization upon the distribution of these antioxidants will be discussed.

M11. Properties of interesterified butteroil. H. D. WEIHE AND G. R. GREENBANK, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

For use in cocoa butter and in some baked foods, a butter with modified physical properties is desirable. Research in chemical modification of other fats indicates that similar treatment of butter might result in the desired product. Butteroil was interesterified in an atmosphere of nitrogen under conditions leading to random interesterification at 55 and 85° C., using either sodium methylate or sodium potassium alloy as catalyst. Directed interesterification was carried out at from 25 to 30° C., using the latter catalyst. Changes in the physical properties of the butteroil as a result of random and directed interesterification are given, respectively, in the following data: capillary melting point from 34.0 to 43.0 and 44.0°; micropenetration (units of one-tenth mm.), at 10° C. from 27.6 to 19.7 and 18.0, at 15° C. from 43.0 to 22.6 and 24.2, and at 20° C. from 62.0 to 58.0 and 33.0; per cent solid phase by dilatometry at 10° C. from 45.6 to 54.4 and 32.2, at 25° C. from 12.2 to 22.0 and 30.5, and at 40° C. from 0.1 to 1.4 and 14.4. The interesterified butteroils were free of off-odors, but even after washing with warm water and heating for 1 hr. at 100° C., with nitrogen at 5 mm. pressure passing through it, the oil gave a slightly bitter flavor when homogenized with fresh skimmilk. The causes of, and means for eliminating, this flavor are being studied.

M12. Characterization of the volatile and nonvolatile fatty acids obtained by a Reichert-Meissl-Polenske distillation. ARUN SENGUPTA,

W. F. SHIPE, AND A. C. DAHLBERG, Cornell University, Ithaca, N. Y.

The fatty acids from butterfat and admixtures of butterfat with other fats were separated by the conventional Reichert-Meissl-Polenske distillation. The volatile fatty acids were separated by chromatographic analysis. The Reichert-Meissl fraction contained butyric, caproic, and caprylic acids. Caprylic and capric acids were the principal components of the Polenske fraction, but it also contained some higher and lower acids. The nonvolatile fatty acids were recovered from the distillation residue by transferring it to a 50% cream-test bottle and centrifuging in a heated Babcock centrifuge. An aliquot of the fatty acids was fractionated with urea. The refractive indices of the original fatty acids, and the fatty acids in the filtrate and precipitate from the urea treatment, were determined. The refractive indices of these three fractions provided a basis for identifying the type of fat. A comparison of the Reichert-Meissl and Polenske values with the refractive indices of the nonvolatile fraction may serve to detect lower levels of adulteration than can be detected by any single value.

M13. Vitamin B_{12} in whey and casein fractions of milk separated by coagulation with acid and rennet. E. B. COLLINS AND MAKOTO YAGUCHI, University of California, Davis.

Vitamin B_{12} was determined by the U.S.P. microbiological assay method, using *Lactobacillus leichmanii* ATCC 7830 and electrometric titration to pH 7.0. Before samples to be tested were diluted to the desired volume, they were adjusted to pH 6.0. Wheys were prepared from pasteurized skimmilk that contained 0.43 γ of vitamin B_{12} per 100 ml. Coagulation with glacial acetic acid produced whey containing 0.19 γ of vitamin B_{12} per 100 ml.; coagulation with rennet produced whey containing 0.28 γ per 100 ml. These amounts were 44 and 65%, respectively, of the amount of vitamin B_{12} in the original skimmilk. The total solids contents of the wheys were 6.78 and 6.86%, respectively. Examination of two additional acid wheys, three cottage cheese wheys, two additional rennet wheys, and two Cheddar cheese wheys indicated distributions of vitamin B_{12} similar to those above. Adjustment of rennet whey to pH 4.6 did not change its vitamin B_{12} content. The larger amount of vitamin B_{12} in rennet whey suggests that rennet may cause slight hydrolysis and/or separation of bound vitamin B_{12} from casein.

M14. Observations on bulk milk quality on truck vs. individual farm tank samples. H. V. ATHERTON, University of Vermont, Burlington.

The quality of farm bulk-cooled milk from individual bulk tanks has been compared with that of samples taken of the combined deliveries in the bulk-pickup truck, using several common laboratory procedures. Studies were made on 13 truckloads of milk, representing 88 tank collections on seven different routes. The weighted average of values determined for individual farm samples was compared with the results of samples taken from the truck at the milk plant. Results showed that the milk flavor (primarily feed) and protein stability were not changed by conditions of pickup. Standard plate, heat resistant, and psychrophilic counts were generally higher on the truck sample than the weighted average of individual deliveries would indicate, but these differences were never bacteriologically significant. This study indicated milk temperatures will increase by from 1 to 2° F. during transport from farm to plant. It would appear that creaming ability, already lowered by conditions of bulk cooling, was further depressed by pumping, and transport in the truck.

M15. Quality of manufacturing-grade milk handled in farm bulk tanks. W. S. LAGRANGE AND F. E. NELSON, Iowa Agricultural Experiment Station, Ames.

Manufacturing-grade milk from 106 producers was examined for standard plate count at 32° C., count of psychrophilic bacteria, thermoduric count, reazurin reduction class, and organoleptic score. Of the 232 samples, 74 had plate counts exceeding 2,000,000/ml and 56 others had counts in excess of 500,000/ml. The count of psychrophilic bacteria frequently was nearly as high as the standard plate count and sometimes exceeded it considerably. Occasionally, the thermoduric count exceeded 10,000/ml. The resazurin reduction test failed to detect many samples with high plate counts. In only a few instances were organoleptic defects attributable to high counts of bacter. Of 62 farms visited, only ten had both a stationary wash-vat and a water heater. Plate counts on only three of the 14 samples from these ten producers exceeded 500,000/ml and only one count exceeded 2,000,000. Among the producers without these two facilities, only a few were able to consistently produce milk with plate counts below 500,000/ml. Other shortcomings in facilities and management were common. Bulk tanks alone are not the answer to quality of manufacturing-grade milk.

M16. Observations on bacteria counts of farm bulk-cooled milk. H. V. ATHERTON, University of Vermont, Burlington.

Recently discussions of milk quality have emphasized the role of psychrophilic and facultative-psychrophilic bacteria in the quality control of milk handled under the bulk-cooling system. Several incubation procedures are used in the dairy industry to determine the bacterial count of farm bulk-cooled milk. The current edition of Standard Methods permits two different media for the Standard Plate Count. There is a need to ascertain whether the several procedures for obtaining bacterial counts on

bulk milk give equivalent results throughout the period of raw storage. Twenty-four samples of bulk-cooled milk were divided into eight replicates for daily study during six days of storage at 40° F. Analyses were made following incubation at 32° C. for 48 hr., 20° C. for 48 hr., 20° C. for 72 hr., 5° C. for seven days, and 5° C. for ten days, using each of the media approved in Standard Methods (tenth edition). Results of this study indicate that counts made on milk samples of a given age are quite comparable, regardless of the plating medium or temperature of incubation. There is no advantage to incubating plates at 5° C. for ten, rather than seven days, or at 20° C. for three, rather than for two, days.

M17. Preserving the activity of lactic cultures. BURDET HEINEMANN, Producers Creamery Co., Springfield, Mo.

To both cheese and butter cultures, 0, 10, and 20% sterile glycerine was added after ripening. The cultures were then held at 35, 5, and −20° F. for periods up to 6 mo. Upon removal from storage, the cultures were used to inoculate autoclaved milk at a level of 1%, and titratable acidity was measured after incubation for 16 hr. Cultures containing 20% glycerine remained active up to 2 mo. of storage at 35° F. and up to 6 mo. of storage at 5 and −20° F. The activity of cultures containing no glycerine was appreciably decreased during the same storage conditions.

M18. Production, distribution, and use of frozen, active lactic acid cultures. J. C. SIMMONS AND D. M. GRAHAM, Clemson Agricultural College, Clemson, S. C.

A procedure which minimizes contamination, eliminates daily transfers, and offers an active culture that will possess uniform characteristics has been studied. The procedure for using these cultures consists of inoculating a suitable medium with an active culture, freezing the culture immediately in a −20° F. room, thawing the culture in a 70° F. water bath, incubating for 16 hr. at 70° F., then using this coagulated mother culture to prepare bulk starter. Studies of these cultures indicate that the organisms produce normal levels of acid and flavor in a medium of nonfat dry milk solids reconstituted at the rate of 10%. If the medium is enriched by the addition of extra milk solids or 10% VS Juice, an increase in acid production is observed. Four commercial starters were frozen, stored at −20° F. for one month, reactivated, and tested for activity by the Leber resazurin and Horrall-Elliker acid production tests. All strains responded equally well when reactivated following storage at −20° F. No difference in activity was observed between the reactivated frozen cultures and the cultures transferred daily. Several of these frozen cultures, used in preparing bulk starter for cottage cheese, developed acidity in the cheese comparable to bulk starter prepared with fresh cultures. The procedure and finished cheese were normal by all criteria.

M19. Effect of temperature on acid production by Streptococcus lactis and Streptococcus cremoris. J. WOLK AND R. P. TITTSLER, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

The amounts of acid produced by nine strains of *Streptococcus lactis* and 14 strains of *Streptococcus cremoris* at different temperatures and combinations of temperatures from 82 to 105° F. were determined. Activity tests were made in sterilized skimmilk for 6 hr. The temperature was held constant throughout, or held constant for a definite period and then raised quickly to one of the higher temperatures. Acid production was measured every 2 hr. either by titration or by pH determinations. Production of acid by both species was greatest at 94° and much less at 100 and 105° F. Some variation was noted among strains of both species. The results emphasize a need for properly adjusting starter-temperature relationships in cheese-making and provide useful data for establishing such relationships. They also show that the use of two temperatures may be more desirable than one in conducting activity tests.

M20. Acceleration of cottage cheese making by using starter culture stimulants. M. L. SPECK AND R. A. LEDFORD, North Carolina State College, Raleigh.

Earlier reports have shown that an aqueous extract of pancreas tissue contained peptides that stimulated the development of various lactic acid bacteria in milk. The present study concerns an extension of the work to commercial types of milk fermentations. Cottage cheese was made from nonfat dry milk solids reconstituted to 10.5% solids in 2-liter batches, using 5% inoculum, 0.02% $CaCl_2$, rennet (1 ml/1,000 lb. milk), and an incubation temperature of 90° F. The time required for the milk to coagulate and reach proper acidity for cutting was reduced approximately 17–41% with concentrations of 0.015–1.0% of pancreas extract solids added to the milk. Concurrent with the decrease in ripening time was a decrease in the cooking time of 12–92%. Similar results were obtained using 30-gal. batches of milk. The cottage cheese made with stimulants had no foreign flavors and had a shelf-life comparable to cheese made with unfortified milk.

M21. The influence of stimulatory peptides on proteolysis of milk by lactic streptococci. M. L. SPECK AND W. T. WILLIAMSON, North Carolina State College, Raleigh.

Numerous studies have shown that the growth of many lactic streptococci in milk are stimulated by various peptide-rich extracts. The role of peptides in stimulating growth has re-

mained unexplained. In the present study, mixed-strain starter cultures showed less proteolysis in milk when the milk was fortified with stimulatory peptides, such as contained in pancreas extract. There was an inverse relation between the amount of proteolysis and the concentration of the pancreas extract in the milk. In addition, the curd tension measurements of cultures at 0.85% titratable acid were greater in milk fortified with pancreas extract than in unfortified milk. The data indicated that the presence of stimulatory pepttides in milk precluded the necessity for cultures to hydrolyze milk proteins before initiating growth. The relation of this study to certain cheese-making steps will be discussed.

M22. Inhibitory effect of nisin upon various organisms. K. M. SHAHANI, University of Nebraska, Lincoln.

Two nisin-producing strains of *Streptococcus lactis* varied in their ability to produce antibiotic activity. In a milk medium containing the nisin-producing organisms, acid production by two strains of *Streptococcus thermophilus* was delayed, but their total acid production was not affected appreciably. Nisin did not inhibit a mixed *Streptococcus* and *Lactobacillus* culture, and two strains each of *S. lactis* and *S. thermophilus*. Both cultures of the antibiotic-producing organisms inhibited only two of 11 strains of *Staphylococcus aureus*. The antibiotic was found to be active only against the coagulase-negative and weak coagulase-producing *Staphylococci*. Nisin was less inhibitory to dairy cultures than penicillin, streptomycin, aureomycin, or erythromycin.

M23. A test for detecting lactic bacteriophage using 2,3,5-triphenyltetrazolium chloride. B. J. LISKA, University of Florida, Gainesville, AND H. E. CALBERT, University of Wisconsin, Madison.

2,3,5-Triphenyltetrazolium chloride (TTC) was used to develop a test for detecting bacteriophage inhibition of lactic cultures. The principle of the test is based upon the inhibition of microbial reduction of TTC by the bacteriophage. Bacteriophage action is indicated when inhibition of normal TTC reduction by the lactic cultures occurs. Controls, known to be free of bacteriophage, are established to determine the degree of inhibition. The results obtained using the TTC test were compared to results using an accepted litmus milk test requiring 12–16 hr. to perform. The TTC test in 4.5 hr. time yielded results comparable and in some cases better than the litmus milk test. Phage was detected in filtrate dilutions up to 10^{-10} or having a titer of ten with the TTC test, when using single-strain *Streptococcus lactis* cultures and specific phage strains. When using multiple-strain lactic cultures, some cases of phage inhibition detected after 4.5 hr. of incubation with the TTC test were not evident after 12–16 hr., using the litmus milk test. Several factors which affect the sensitivity of the TTC test are: *(1)* amount of inoculum; *(2)* concentration of TTC; *(3)* time of TTC addition, and *(4)* length of time for the test.

M24. Association between Bacterium linens and an oxidative type yeast in the ripening process of surface-ripened cheeses. R. L. MAGINNIS AND J. F. CONE, The Pennsylvania State University, University Park.

A study was made of an endocellular polypeptidase extracted from an oxidative type yeast (*Trichosporon* species) isolated from Trappist-type cheese. This enzyme was capable of further breaking down at TCA soluble polypeptide formed by the action of *Bacterium linens* on a 2.5% casein medium prepared by the methods of Peterson (1948). A crude autolysate containing the yeast polypeptidase was partially purified by precipitation of the active fraction with two volumes of ethanol to one of autolysate. The activity of the enzyme on the polypeptide preparation was measured by titration with alcoholic HCl of the enzyme-treated substrate in acetone, according to a modification of the Linderstrom-Lang method. Preliminary studies on pH optimum showed little activity below 6.0. There was a broad peak at pH 6.6 to 7.0. The optimum temperature was 30° C. A study of the effect of activators and inhibitors showed that Zn and Mn ions gave appreciable activation.

M25. Effect of penicillin upon the morphology and gram-staining characteristics of Streptococcus lactis and Streptococcus thermophilus. R. W. BAUGHMAN AND F. E. NELSON, Iowa State College, Ames.

Morphological changes induced by various concentrations of penicillin were studied at temperatures of 21, 32, 37, and 45° C. Morphological changes were more rapid at higher penicillin concentrations. Cells became greatly elongated and rather irregular in shape in the presence of the antibiotic. The cells of *S. thermophilus* tended to form extremely long chains, even in concentrations of penicillin as low as 0.03 unit per milliliter. The typical gram-positive staining reaction of *S. lactis* and *S. thermophilus* was reversed upon exposure of the cells to various concentrations of penicillin. As the concentration of the penicillin was increased, this phenomenon was more pronounced. However, even at levels as low as 0.05 unit per milliliter this reversal was apparent at 45° C. within one hour when using *S. thermophilus*.

M25A. Taxonomy of carbon dioxide–producing lactic streptococci found in mixed-strain starter cultures. W. E. SANDINE, P. R. ELLIKER, AND A. W. ANDERSON, Oregon Agricultural Experiment Station, Corvallis.

The causes of the floating curd defect in cottage cheese have been studied. It has been

demonstrated that this defect often is caused by high carbon dioxide–producing lactic acid streptococci found in mixed-strain starter cultures. When these types became the dominant organisms in a mixed culture, a high gas-producing starter culture resulted. Single strains isolated from these high gas-producing, mixed-strain starter cultures, on the basis of their rate of acid and carbon dioxide production, were placed in four general groups, namely: high acid–high gas, low acid–high gas, high acid–low gas, and low acid–low gas. A taxonomic study was made properly to classify each one. Known species of lactic acid streptococci and associative *Leuconostoc* organisms that may be used in controlled dairy fermentations were included for comparison with organisms isolated from mixed-strain cultures. Each organism was examined for at least 34 different cultural reactions. The high acid–high gas and the low acid–high gas types were identified as *Streptococcus diacetilactis,* the low acid–high gas types as *Streptococcus cremoris,* and the low acid–low gas types as *Leuconostoc* species or slow acid-producing strains of *S. cremoris. Streptococcus lactis* organisms were not found.

M26. Some observations on lipase activity. B. L. Herrington and E. S. Guthrie, Cornell University, Ithaca, New York.

Many of the factors responsible for induced rancidity in cow's milk have been identified, but less is known about factors responsible for spontaneous rancidity. The stage of lactation does not seem to be a factor affecting induced rancidity, but it may be a factor in spontaneous rancidity. Acid degree values have been determined at regular intervals on the milk of 18 individual cows at zero-time and after 24 hr. of refrigeration. The experiment will continue for entire lactations. It is already apparent that lipase activity is very low in milk secreted during the first week of lactation.

M27. Effect of light, agitation, acids, and rennet upon lipolytic activity in milk. B. J. Demott, University of Tennessee, Knoxville.

Exposure to sunlight for one hour of homogenized milk in clear or amber-colored glass bottles resulted in slightly lower free fat acidities than milk not so exposed. The amber-colored bottle protected the enzyme(s) only very slightly when its activity was determined immediately, but protection was apparent after the milk has been stored 24 hr. at 40° F. The protective effect of the amber bottle against the development of oxidized flavor was demonstrated. Mixtures of pasteurized whole milk and raw skimmilk, one of which was agitated in a Waring Blendor, shows that agitation of the substrate is more effective in producing lipolysis than a like agitation of the enzyme(s). Precipitation of casein by use of HCl, lactic acid, or rennet showed the latter to have the least destructive effect on the lipase(s).

M28. Acid values and 1-monoglyceride content of normal and rancid milk. R. G. Jensen and M. E. Morgan, Storrs Agricultural Experiment Station, Storrs, Connecticut.

This study showed that the 1-monoglyceride content of fat from milk increased as the milk became rancid. Five samples of milk were prepared by mixing raw and homogenized milk in equal portions. The samples were analyzed for acid degree values (ADV) (silica gel method) and 1-monoglycerides, immediately after preparation and after 24 hr. of refrigerated storage. The fat for the monoglyceride analysis was obtained by using a silica-gel extraction method similar to the ADV procedure. The monoglyceride contents were estimated by colorimetric determination of the formaldehyde formed from the oxidation of 1-monoglycerides by periodic acid. The average ADV of the normal samples was 1.67 (1.43–1.90) and the average 1-monoglyceride content (reported as m*M* per 100 g. fat) was 0.304 (0.208–0.418). The average ADV for the 24-hr. samples was 11.56 (10.16–12.31) and the average 1-monoglyceride content was 0.669 (0.510–0.895). Theoretically, there could be 5.78 m*M* of 1-monoglycerides for an average ADV of 11.56. The level of 1-monoglycerides found may be low, because of the presence of diglycerides and 2-monoglycerides (through which fat hydrolysis proceeds with pancreatic lipase). 2-Monoglycerides are not oxidized by periodic acid. The 1-monoglyceride found probably arise from isomerization of 2-monoglycerides.

M29. Distribution and partial purification of milk lipases.* Z. Saito, W. J. Harper, and I. A. Gould, The Ohio State University, Columbus.

Using activities at pH 7.0 and pH 8.6 as indicative of two different lipases, the distribution of these lipases in cream, skimmilk, and sediment obtained by centrifugation at 6,000 × gram for 30 min. has been studied. Lipase activity at both pH levels was observed in all three fractions. However, the relative proportion of activity in the different fractions varied. Slight purification of milk lipases was achieved by centrifugation. The extent of purification varied markedly, according to season of the year and to the individual milk supply. Generally, increases in specific activity of about three- to fivefold of both pH 7.0 and 8.6 lipases were obtained. Butanol extraction, phosphate-gel and ethanol fractionation were utilized for further purification of the milk lipases. Acetone and magnesium sulphate fractionations were not successful in further purification of the milk lipases. Paper chromatographic analysis of the partially purified lipase preparation, using an acetone-water solvent system, revealed separation of lipase ac-

* Supported in part by funds from the Ohio Research Foundation for fundamental research, and by the Ohio Dairy Products Research Fund.

tivity at pH 7 and at pH 8.6. The lipase had Rf values of 0.75 and 0.9, respectively, and were separated from the majority of the other proteins in the preparation.

M30. Rates of lipolysis in milk as affected by duration of activation by shaking. N. P. TARASSUK AND M. YAGUCHI, University of California, Davis.

Freshly drawn milk from individual cows was activated by shaking at 37° C. for periods up to 6 hr. The rates of lipolysis during shaking, and after shaking and aging in cold, were investigated. The velocity of lipolysis is highest during the first hour of activation by shaking, it decreases thereafter, and after 2 hr. of shaking it remains fairly constant at about one-third of its initial (first-hour) rate. This initial rate varies with different milks and accounts for existing variation in susceptibility to lipolysis. The extent of lipolysis in activated milks developed in storage (total F.F.A. titration—F.F.A. titration right after shaking) increases rapidly with the increase in time of activation by shaking, and reaches a maximum value in milk shaken an average of 1 hr. The value thereafter remains nearly constant on shaking for as long as 6 hr. and it is of about the same magnitude for all milks tested. These data, on velocity of lipolysis during shaking, indicate that absorption of lipase on fat globules is complete on activation by shaking for 1 hr.

M31. Possible role of xanthine oxidase in spontaneously oxidized milk. A. E. WOODS, L. W. AURAND, AND W. M. ROBERTS, North Carolina State College, Raleigh.

Studies were made to determine the role of oxidizing enzymes, normally present in milk, in the development of spontaneously oxidized milk. Xanthine oxidase, peroxidase, catalase, and their substrates, in varying concentrations, were studied. The milks used for this study were raw, batch-pasteurized, and heat-inactivated (76.6° C. for 30 min.). Results of these studies show that peroxide and catalase, together with varying levels of substrate, had no effect on the development of oxidized flavor. Conversely, xanthine oxidase when added at optimum levels of activity, gave rise to an oxidized flavor. Hypoxanthine in varying concentrations prevented the development of the off-flavor. Thus, an enzyme mechanism is suggested; namely, xanthine oxidase acting upon an unknown substrate(s) gives rise to the oxidized flavor principle.

M32. Study of the phosphatase test for milk pasteurization. J. B. MICKLE, P. J. MAGNINO, JR., AND R. D. MORRISON, Oklahoma State University, Stillwater.

Milk was pasteurized at 62.5 and 76.7° C. and tested for phosphatase activity immediately after pasteurization and again after 24 hr. of incubation at 32.2° C. In Trial I, the phosphatase test was run in the customary manner. Trial II was a repetition of Trial I, except that a bioassay technique was used in analyzing the samples. The minimum time of pasteurization was determined at each heating temperature and this was found to vary. This was particularly true at 76.7° C., where the minimum pasteurization time was 1 min., 56 sec. in August, 1957, and increased to 2 min., 6 sec. during December, 1957.

Phenol values of 3.6 ± 1.3 p.p.m. were established as the minimum for milk pasteurization in Trial I. In this trial, three of 16 samples appeared to be pasteurized after heating, but according to the phosphatase test were underpasteurized after incubation. However, no general trend toward higher or lower phosphatase values after incubation was established. In Trial II, ten of 13 samples gave higher values after incubation. However, these increases were usually smaller than the experimental error of the first trial.

M33. Stability of disodium phenyl phosphate in carbonate-bicarbonate buffer. R. W. HENNINGSON, Clemson College, Clemson, S. C.

A major practical impediment to the routine use of any phosphatase method is the instability of the disodium phenyl phosphate. Samples of this substrate were prepared in carbonate-bicarbonate (Cornell) buffer and treated and stored in the following manner: Sample No. 1 was unheated, $CHCl_3$-preserved, and stored at 20° C., No. 2 was unheated and stored at 5° C., No. 3 was unheated and stored at 20° C., No. 4 was heated to 85° C. for 3 min. and stored at 5° C., No. 5 was heated to 85° C. for 3 min. and stored at 20° C., No. 6 was autoclaved at 120° C. for 10 min. and stored at 5° C., and No. 7 was autoclaved for 10 min. at 120° C. and stored at 20° C. Samples were removed from storage at regular intervals and tested by adding N,2,6-Trichloro-*p*-quinoneimine (CQC) to the sample. Samples 1 and 3 showed 10 γ phenol after 1 wk. Sample No. 2 showed 5 γ after 2 wk. None of the other samples showed any phenol after 1 yr. of storage. At this time, a fresh buffer substrate was prepared and tested with stable, stored samples, using 0.04% raw milk, for sensitivity. All showed 8 γ phenol. Samples 1, 2, and 3 showed greater than 40 γ phenol after 1, 4, and 2 mo. of storage, respectively. Heated buffer-substrates used routinely over a period of 1 yr. showed no instability, when care was used in pipetting. Results indicate that heating the buffer-substrate maintained sensitivity, and kept it in a stable form for routine use, eliminating the necessity for preparing fresh solutions each time tests were to be made.

M34. A method to improve the sinkability of dried whole milk. D. H. BULLOCK AND W. C. WINDER, University of Wisconsin, Madison.

A simple test has been devised to estimate the

per cent of a milk powder sample which sinks after being placed on a quiescent water surface for a given period of time, usually 30 sec. Results are expressed as per cent sinkability. Studies to improve this sinkability property have been conducted on conventional whole milk powder and, in particular, on high-vacuum dried whole milk. Whole milk powder, in general, exhibits greatest sinkability when first removed from the drier. Much of this property is lost during the first day or two of storage. By subjecting the powder to a specific post-drying temperature treatment, the initial sinkability of the fresh powder could not be retained but could be improved considerably. The following whole milk powders exhibited marked increases in sinkability after the temperature treatment. The tests were made 48 hr. after the treatment. High vacuum–dried powder increased from about 20% to from 70 to 90%; spray dried powder, from 10 to 40%; aggregated spray-dried powder, from 17 to 70%, and roller-dried powder, from 7 to 17%. Subsequent tests on high vacuum–dried powder showed that the property was not lost, even after months of storage.

M35. Effect of raw milk quality on the flavor stability of dried milk. U. S. Ashworth and C. C. Prouty, State College of Washington, Pullman.

Experimental lots of spray-dried whole milk powder were prepared from raw milk having poor quality due to the presence of the following types of bacteria: *(1)* acid formers, *(2)* proteolytics, *(3)* lipolytics, and *(4)* psychrophiles. After preheat treatments of 175° F. for 10 min., the samples were spray-dried along with controls from the same bulk milk. The powders were stored at 45 and 100° F., both air- and nitrogen-packed for a total of 8 mo. Sample cans were taken from storage each 2 mo. for flavor evaluation and for chemical and bacteriological testing. The results from 170 lots of powder show that stale flavor develops in the powders, independently of the quality of the raw milk, and overshadows most flavor defects resulting from drying poor-quality milk within certain limits. In the freshly prepared powders after reconstitution, the flavor panel could significantly detect a drop in pH of 0.2 unit caused by bacteria. It could also detect proteolysis sufficient to give free tyrosine values over 1 mg. per g. of milk protein and lipolysis, sufficient to produce a value of two for the free fatty acid titer. The temperature of storage of the powders had a greater influence on flavor stabiltiy than did the bacterial quality, as measured by the standard plate count or by differential counts for acid formers, proteolytics, lipolytics, and psychrophiles.

M36. Identification of some compounds affecting the flavor of nonfat dry milk. Richard Bassette and Mark Keeney, University of Maryland, College Park.

Nonfat dry milks (NFDM), particularly some of the instant dry milks, are sometimes criticized for having cereal, hay, malty, and stale types of flavor. Study of the volatiles recovered by low-temperature vacuum distillation of reconstituted instant NFDM demonstrated that carbonyl compounds and methyl sulfide played a prominent role in these off-flavors. The following carbonyl compounds have been identified via their 2,4-dinitrophenylhydrazones: diacetyl, furfural, acetaldehyde, acetone, 2-methylpropanal, 3-methylbutanal, hexanal, and nonanal. Several other volatile carbonyls are present in cereal-stale flavored NFDM and evidence is being accumulated for their identification. The compounds thus far identified substantiate previous suggestions, that Maillard-type browning is involved in cereal flavor development. The mechanism of flavor development will be discussed in light of the identified compounds.

M37. Further investigations on the stale-flavor components in dried whole milk. W. W. Nawar, H. E. T. Dall, A. S. Ganguly, S. H. Lombard, and R. McL. Whitney, University of Illinois, Urbana.

The CCl_4-vapor distillate of stale butteroil extracted from stale dried whole milk contains stale-flavor components, and its infrared spectrum indicates the presence of carbonyls. Therefore, an extraction of stale butterfat with Girard's-T-reagent was successfully attempted. Attempts to further fractionate the Girard's-T-extract by alkaline or sodium-bisulfite extraction resulted in loss of the stale flavor. To determine whether alkalinity, as such, would prevent the development of stale flavor, dried whole milks prepared from condensed milks adjusted to different pH levels were stored under varied conditions. Alkalinity was found to delay the appearance of stale flavor; however, even at pH 7.5, the alkaline flavor frequently was detected by the judges. Further work on the fractionation of the stale-flavor components revealed that there are at least two different stale-flavor components: one, Girard's-T-extractible but nonvolatile, and the other, volatile but non-Girard's-T-extractible. The relative distribution of the stale flavor between thees two components seems to vary with the character and previous history of the dried whole milk under investigation. The Girard's-T-extractible-nonvolatile fraction has been further fractionated by paper chromatography into a stationary and a mobile component, both of which contain stale-flavor components.

M38. Migration of phosphatides in processing foam- and spray-dried whole milk. G. R. Greenbank, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Many investigators who have studied the oxidative deterioration of dry whole milk have proposed that the phosphatides trigger the oxidation of the glycerides. In further experiments on the relationship of milk phosphatides to the stability of dry whole milk, data are reported that show the phosphatides (as measured by those phosphorus-containing materials which are soluble in fat solvents) migrate from the fat phase (cream) into the water phase (nonfat milk) as processing proceeds through pasteurization, condensation, homogenization, and drying. Any form of agitation causes the phosphatides to move from the fat globules into the skimmilk. In some batches of foam-dried whole milk the nonfat phase of the reconstituted milk contained twice as high a concentration of phosphatides as was found in the control milk. The data are discussed with regard to the light they throw on processing methods which have been found by these laboratories and industry to retard deterioration. A new and simple method of comparing the phosphatide content of different samples of butterfat is described.

M39. Effect of superheating on the baking properties of nonfat dry milk. L. V. ROGERS, H. A. ANDERSON, AND C. F. HUFNAGEL, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

In preparing nonfat dry milk (NFDM) especially for use in baking, skimmilk normally receives a high-temperature forewarming treatment, as this gives a product with good baking properties. However, since NFDM has varied uses and a wide range of conditions prevail in its manufacture, a study was made of the effect of these conditions, especially superheating, on the baking properties of spray-dried NFDM. To cover the range of commercial practices, three forewarming temperatures, three solids concentrations in the condensed skimmilk, and three superheating conditions were used. The NFDM was baked into white pan-bread at the 6% level, based on the flour, and the effects of the processing steps on such factors as water absorption of the dough and loaf volume were determined. In general, undenatured whey protein nitrogen in the NFDM decreased with the extent of the forewarming treatment of the skimmilk and the severity of superheating of the concentrate following low forewarming of the skimmilk. The baking properties of NFDM receiving the optimum forewarming treatment were not further improved by superheating of the skimmilk concentrate. Superheating the skimmilk concentrate for 30 min. at 175° F. imparted satisfactory baking properties to the NFDM, where an inadequate forewarming treatment was applied.

M40. Free fat in foam-dried whole milk. A. TAMSMA, L. F. EDMONDSON, AND H. E. VETTEL, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

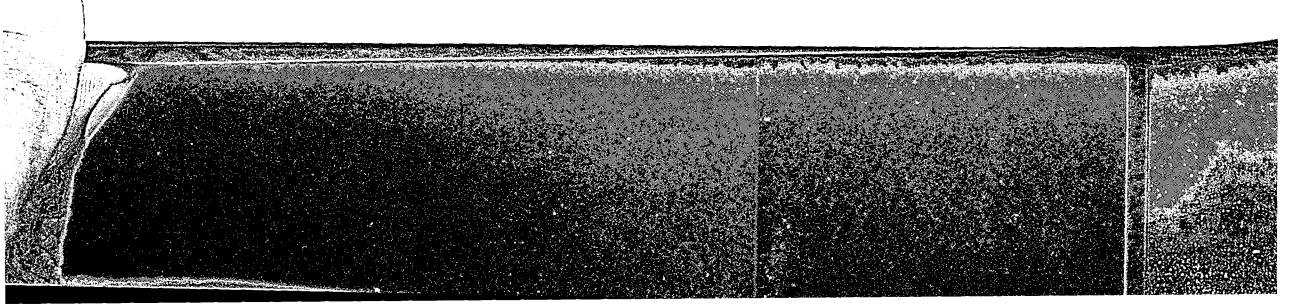
Concentrated milks of 50% T. S. were foamed and dried in a vacuum shelf drier, and comminuted through a 20-mesh screen. Methods were developed for determination of free fat by extraction with carbon tetrachloride and of that fat readily separated by centrifugation of the reconstituted product. Large increases in free fat of from 10 to over 90% of the total fat resulted from aging and lactose crystallization of the concentrate. This effect was reversed by heating immediately before drying. Free fat decreased slightly with increased homogenization pressure. Free fat contents less than 10% of the total fat were obtained by control of these factors. Variations in free fat up to about 40% did not affect dispersibility of the dry milk or the state of dispersion of the fat in reconstituted milk. At high free-fat levels (40–95%), the dispersibility decreased and part of the fat was readily separated by centrifugation. Free fat increased with decrease in particle size. A fraction obtained by comminution through a 40-mesh screen, followed by removal of the finest particles by sieving on a 60-mesh screen, was similar in free fat, more dispersible, and much higher in bulk density, when compared to the product obtained by comminution through a 20-mesh screen.

M41. High milk-protein bread. L. V. ROGERS AND HELEN H. WELTON, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Satisfactory bread containing 25% nonfat dry milk based on the flour weight, or 20% by content, has been experimentally produced without major changes in customary bread production. The bread is typical of white pan-bread, except for a firmer crumb and a heavier and darker crust. Loaf volume is about normal. Taste evaluations showed this bread to have an outstanding milk flavor and excellent palatability. Nutritional studies are incomplete, but excellent protein quality is expected.

M42. A study of the processes of gelation, flocculation, and caramelization as related to heat stability in concentrated milk. R. N. GIROUX, Ecole de Laiterie, Provinciale, St. Hyacinthe, Que., AND H. E. CALBERT AND A. M. SWANSON, University of Wisconsin, Madison.

Ultra-high temperature sterilization gives products of better flavor and color; however, gelation and fat separation are limiting factors. Using the proposed method, these factors were found to be associated with the heat stability phenomenon. The accelerated gelation-flocculation-caramelization test (G.F.C.) is based on the observation, by a procedure described in Paper M49, 52nd Annual A.D.S.A. meeting, Stillwater, Oklahoma, 1957, of viscosity changes upon quiescent heating of concentrated milk.



The results are presented in the form of a graph, relating viscosity to time of heating. The typical G.F.C. curve represents three processes: gelation, flocculation, and caramelization. Gelation is characterized by a reversible gel development up to a high maximum viscosity. Flocculation is characterized by an irreversible destruction of the gel to a minimum basic viscosity and is accompanied, among other things, by the loss of the solid suspension stability. Caramelization, as observed visually, is particularly characterized by an increase in viscosity which progresses at an accelerated rate. The addition of phosphate and calcium salts had a striking effect upon the G.F.C. curve. Although the general pattern remained the same, the intensity of the response was greatly modified.

M43. Ultra-high temperature sterilization and aseptic canning of concentrated milk with pilot equipment. G. H. HARTMAN, White House Milk Co., Inc., Manitowoc, Wisconsin, AND D. V. ALSTRAND, American Can Co., Maywood, Illinois.

Concentrated milk (2-1) was sterilized by a pilot tubular heat exchanger, homogenized at 3,500 p.s.i. after partial cooling, followed by additional cooling and aseptic canning. Samples were evaluated for flavor by multiple comparison procedure, using dilution technique by from ten to 12 judges and by direct scoring, using the proposed A.D.S.A. dry milk scorecard. Fresh canned milk diluted with 50% market milk rated slightly different in flavor from market milk. Preheating variables showed no significant difference in flavor. Reconstituted milk was slightly more viscous, equal in color, and scored several points lower in flavor than market milk. Heated flavors declined after from 8 to 12 wk. of storage at 40° F. and storage flavor developed thereafter. None gelled after 20 wk. Fat separation and precipitates were not detected after 20 wk. for most samples. Samples stored at 80° F. showed a slight storage flavor at 4 wk. Some lots held at 100° F. gelled in 8 wk., but others did not after 20 wk. Viscosity dropped slightly during the first month of storage, then remained constant. This test was useless in predicting gel formation. Milk not homogenized after sterilization was chalky, tactual, and viscous. Flavor stability is the most important factor, if this type of milk is to be marketed for beverage use.

M44. Factors pertaining to the stability of ascorbic acid and vitamin B_6 in modified milk products. O. F. GARRETT, R. W. SHARP, AND JEAN KLECKNER, M & R Dietetic Laboratories, Inc., Columbus, Ohio.

The stability of ascorbic acid and vitamin B_6 in modified infant-feeding milk products has been studied. Processing losses of B_6 in the powder were about 23%. Processing losses of the natural forms of B_6 in the sterilized liquid prior to sterilization were about 14%; sterilization losses were 25% of the natural forms and 10% of the synthetic form of B_6; sterilization losses of ascorbic acid were about 30 to 35%. Storage losses of ascorbic acid in the sterilized liquid were measurable, but B_6 was stable. Internal can surfaces of plain tin, "corn" enamel, and epoxy resin enamel did not influence the stability of ascorbic acid during sterilization and storage of the concentrated liquid, but losses of B_6 were 5 to 10% higher in the enameled cans. In terminally autoclaving the infant-feeding formula, destruction of B_6 was arithmetically proportional to the length of heating and semilogarithmically proportional to the temperature of heating. Losses at 250° F. for 5 min. and at 230° F. for 10 min. were comparable.

M45. Relation of type of fat in modified sterilized milk products to stability of vitamin B_6 and to physiological response when fed to rats. L. S. BAUR AND O. F. GARRETT, M & R Dietetic Laboratories, Inc., Columbus, Ohio.

Differences in the stability of vitamin B_6 in modified sterilized milk products containing six different single fats and one fat mixture were not significant. Three rat-feeding experiments were conducted in which the modified milk products were used. In the first experiment, the modified products contained nutrients that were physiologically balanced for infant feeding in which over 50% of the solids consisted of lactose. The fats in the four products studied were, respectively, milk fat alone, corn oil alone, cocoanut oil alone, and a fat mixture having a 50:50 ratio of saturated and unsaturated fatty acids. In the second experiment, the modified products contained nutrients that were physiologically balanced for rats, in which about 25% of the solids consisted of lactose. The same kinds and level of fats were used in the four modified products as those used in the first experiment. In the third experiment, the modified products contained nutrients that were physiologically balanced for infants. All four products contained the same level of fat as used in the first two experiments. Three of the products contained fat mixtures that varied in content of milk fat and cocoanut oil and the fourth product contained milk fat alone. Multiple levels of vitamin B_6 were used in the products of all three experiments.

M46. Market agency education assists dairy operators in solving engineering and cost problems. A. L. RIPPEN, Michigan State University, East Lansing.

A program in market agency education has been introduced for dairy plant operations in Michigan. Some of the services offered include assistance to improve operating efficiency through consultation on dairy engineering problems and suggested plant layouts. Studies

have indicated wide cost variations between plants in various phases of processing. Some examples of variations in costs of processing steps for 100 lb. of milk are: *(1)* Homogenization costs of 1.3, 5.5, and 10.5¢ for plants processing 80,000, 10,000, and 5,000 lb. per day, respectively. *(2)* Bottle washing costs for 100-bottles-per-minute capacity of from 9 to 16.0¢ for plants handling 100,000 and 20,000 lb. per day, respectively. Suggestions for reducing operating expenses often include more efficient plant layout, improved utilization of labor and equipment, and better processing methods. The trend of plant consolidations will likely continue as a means of increasing volume, with a greater opportunity to reduce units operating costs. Dairy plant operators have responded favorably to the program.

M47. Influence of rapid cooling on the development of a rancid flavor in milk produced by bulk systems. P. E. JOHNSON AND R. L. VON GUNTEN, Oklahoma State University, Stillwater.

The influence of rapid cooling of milk on the activation of lipase was suspected while studying other phases of the rancid-flavor problem in bulk-handling systems. Composite samples of individual cow's milk which had been collected at the discharge end of a pipeline system and allowed to set at milk-room temperature during the milking operation had an average fat acid degree value of 1.63; whereas, corresponding tank samples collected at the end of the milking period had an average fat acid degree value of 3.24. To further investigate the effects of rapid cooling, samples of milk from individual cows were collected at the discharge of a pipeline system and divided into two portions. One portion was cooled immediately, and cooling was delayed on the other portion. At the end of approximately 48 hr., the average fat acid value of the slowly cooled samples was 1.65, whereas on the rapidly cooled samples it was 2.70. In farm operations where rancid flavor was a problem, on alternate pickup days the refrigeration on the tank was not turned on for approximately 1 hr. after the milking began. On the other alternate pickup days, normal cooling was practiced. The average fat acid value on the milk from these farms was 1.69 when the cooling was delayed. Under normal cooling operation the average fat acid value was 2.36.

M48. A radiometric method for measuring stainless steel corrosion. W. J. HARPER, The Ohio State University, Columbus.

Under acidic conditions, radioactive phosphorus will react irreversibly with the surface of stainless steel. This reaction has been utilized to provide a basis for a rapid quantitative method for measuring stainless steel corrosion. Two to three microcuries of P^{32} in $N/1$ H_3PO_4 were applied to 24 cm.^{2} of the surface of 2- by 3-in test strips of stainless steel. The strips were dried at 100° C. for 30 min. The unreacted P^{32} was removed by a standardized washing procedure and redried at 100° C. for 30 min. The strips then were totally immersed in the corrosive test solution at room temperature and after 12 hr. the amount of P^{32} removed from the stainless steel was measured. The concentration of P^{32} provided an index of corrosion. Sodium chloride and various cleaners and sanitizers were used as test solutions with stainless steels of the 200, 300, and 400 series. The data obtained were in relative agreement with published results, using other methods of measuring corrosion. Comparison was made between the radiometric method and the loss of weight after total immersion for 60 days at room temperature. The results of the two methods agreed in magnitude with various test solutions.

M49. Factors of value design associated with the homogenization of milk.* J. J. BETSCHER, W. J. HARPER, I. A. GOULD, AND D. A. SEIBERLING, The Ohio State University, Columbus.

An experimental homogenizer valve assembly was designed and constructed to permit variation of some of the factors associated with homogenization. The time required for a particle to pass through the valve clearance, the velocity through the clearance, the entrance and exit conditions of the valve, and the effect of two or more valves in series were studied. Valve performance was evaluated in terms of both operating characteristics and homogenization efficiency. The data indicated that the efficiency of homogenization was directly related to the velocity through the valve clearance. Neither the efficiency of homogenization nor pressure varied with valve length. With a valve which was modified by beveling the internal diameter at one of the ends, the pressure drop and homogenization efficiency were affected when the beveled end was used as the valve entrance. However, when the beveled end was used as the valve exit, only the efficiency of homogenization was affected. In multiple stage valves the efficiency of homogenization appeared to be more closely related to the total pressure drop than to the number of valves in series.

* Supported in part by funds from the Ohio Dairy Products Research Fund.

M50. Influence of bias in different age groups on consumer preferences of chocolate drink. H. S. WILLARD AND W. R. THOMAS, University of Wyoming, Laramie.

To find out if age of the consumer alters the bias produced by the serving order of chocolate drink, between 20 and 30 participants of both sexes in each of the fourth, fifth, and sixth school grades, and the same number of students of college age, were given three samples successively of chocolate drink per session, and

were asked to express the degree of likes and dislikes on a Hedonic scale. Each drink was identical except for the brand of cocoa. Fourteen different brands were used, with one brand of cocoa as control appearing at each session, the two other brands being different. Orders of serving were altered in the following ways: *(1)* Same order in all grades, *(2)* order reversed in all grades, *(3)* order changed between each grade, and *(4)* order changed within each grade. The average scores declined with the order of serving in each grade, but with lesser degree from the fourth to the college grade. Statistical treatment of the data by determining the least significant differences indicated that differences between the first and third serving were statistically significant at the 5% level in the fourth, fifth, and sixth grades, but not at the college level.

M51. Production of a high-nitrogen, low-lactose product from whey using an automatic pH control system. D. R. ARNOTT, Purdue University, Lafayette, Indiana, AND S. PATTON AND E. M. KESLER, The Pennsylvania State University, University Park.

A product with increased nitrogen content has been produced from fermented Cheddar cheese whey treated with anhydrous ammonia. The ammonia was added continuously with an automatic pH control device. The pH of the whey was lowered to approximately 5.0 with *Lactobacillus bulgaricus*. This pH level was maintained throughout the fermentation period. Nitrogen levels in excess of 0.80% were achieved in less than 15 hr. of fermentation with a 10% inoculum. The lactose content was lowered to less than 1.0%. The ammoniated whey was concentrated to approximately 40, 50, 60, 80, and 90% total solids. Analyses of the concentrated whey included fat, lactose, ash, and lactic acid. Nitrogen loss durnig concentration ranged from 4.33 to 8.93% of the total nitrogen present. The concentrated whey stored at room temperature for 153 and 365 days showed a maximum nitrogen loss of 1.0% for the 365-day period. This product may have value as a ruminant feed, as a fertilizer, or as a fermentation additive.

M52. The manufacture of improved quality sherbets. W. S. ARBUCKLE AND E. A. DAY, University of Maryland, College Park.

A survey of commercial sherbets indicated a general lack of quality when compared to the ice cream marketed by the same manufacturers. Consequently, investigations were conducted to determine the effect of composition on the freezing point, handling properties, body and texture, total acidity, and consumer preference. The physical properties of the sherbets were controlled primarily by both the quantity and the type of sweetener used, along with a constant amount of stabilizer. Sweeteners used were sucrose, dextrose, and three corn syrup solids (C.S.S.) preparations with dextrose equivalents (D.E.) of 30, 42, and 54. The low conversion (30 D.E.) C.S.S. was most satisfactory when used with sucrose (1:1) to a total of 26% sweetener solids, for obtaining sherbets with a smooth texture and chewy body. This sherbet had a freezing point of 28° F. and exhibited superior handling properties. The other C.S.S. preparations were required in higher concentrations to achieve the same effect on body and texture, but resulted in a substantial lowering of the freezing point. A total acidity of from 0.55 to 0.60% was most desirable in practically all sherbets studied. The significance of variation of composition on caloric values and the results of consumer preference tests will be discussed.

M53. A centrifugal ultrafiltration method for studying the soluble constituents of ice cream. B. W. THARP AND P. G. KEENEY, The Pennsylvania State University, University Park.

Ultrafiltration of ice cream through cellulose-dialyzing membranes in a refrigerated centrifuge at temperatures above 14° F. gave sufficient yield of ultrafiltrate for analysis. To determine if ultrafiltrates obtained by this method were representative of the unfrozen portion of the ice cream, comparisons were made, using ultrafiltrates of ice cream and ice cream mix, at 21 to 28° F. Calcium content, freezing point, and refractive index were used as the basis for these comparisons. The calcium content and refractive index of ice cream ultrafiltrates increased, and freezing point decreased, as temperatures of ultrafiltration were lowered. The magnitude of these changes was in accordance with accepted theories concerning concentration of soluble constituents in the unfrozen portion of ice cream. Using the data collected in this study, calculated values for the amount of water frozen at various temperatures closely agree with theory, as well as with values obtained at similar temperatures by another investigator. Therefore, these ultrafiltrates are representative of the unfrozen portion of ice cream.

M54. Influence of selected ingredients on the melt-down of vanilla ice cream. P. J. ROBERTS AND D. R. FRAZEUR, Purdue University, Lafayette, Indiana.

Experiments designed to determine the influence of certain ice cream ingredients on the melt-down of vanilla ice cream were conducted, using standardized methods of mix preparation, freezing, holding, and melting. A standard mix composed of 12% milk fat, 11% milk solids-not-fat, and 15% sucrose was used wherever applicable. Stabilizers and emulsifiers were used at manufacturers' recommended levels. Satisfactory ice cream melt-downs were obtained with sodium alginate, Irish moss extract, and car-

boxymethyl cellulose either with or without emulsifiers. Guar seed gum, locust bean gum, gum karaya, and gelatin did not yield satisfactory melt-downs unless used with emulsifiers. It was found that in certain cases, the curdiness caused by a particular stabilizer could be improved by one emulsifier, but another emulsifier migh have little or no such action. The degree of curdiness observed was inversely related to the rate of melt-down. The addition of 0.15% disodium phosphate or sodium citrate had less effect on melt-down than did the optimum emulsifier. The substitution of corn syrup solids for part of the sucrose caused curdiness in proportion to the amount added.

M55. Interrelationships of heat treatment, solids-not-fat, and calcium chloride to the yield of cottage cheese. C. W. Dill, W. M. Roberts, and R. J. Monroe, North Carolina State College, Raleigh.

The effects of pasteurization temperature, solids-not-fat content, and the addition of varying amounts of calcium chloride to milk after pasteurization on the yield of cottage cheese were studied. All three variables affected the yield of cottage cheese expressed as pounds of cheese per pound of solids-not-fat in the skimmilk. Fortification of the skimmilk with nonfat dry milk to a solids-not-fat content of from 10 to 11% increased the yield of cottage cheese per pound of solids-not-fat. Increases in pasteurization temperature resulted in progressive increases in the yield of cottage cheese over the entire temperature range studied. The addition of varying amounts of calcium chloride to pasteurized skimmilk affected the yield of cottage cheese only slightly when considered separately, the main effect from calcium chloride being its desirability in the presence of increased heat treatment and/or increased solids-not-fat content. There were significant interactions between the three variables studied.

M56. Effect of skimmilk heat treatments on cottage cheese manufacture. D. B. Emmons, A. M. Swanson, and W. V. Price, University of Wisconsin, Madison.

Defects in cottage cheese from nonfat dry milk have been attributed to overheating skimmilk during processing. Defects in conventionally manufactured cottage cheese occur when skimmilk is heated sufficiently to alter the whey proteins. When skimmilk is heated to 175° F. for 30 min., the combined use of 20 ml. of rennet per 1,000 lb. of skimmilk and of the A-C test restores the moisture-expelling properties of the curd and minimizes fragility. Recovery of skimmilk solids as curd is increased approximately 10%. Critical judges find a certain amount of mealiness and unevenness in body when the curd is cooked and washed conventionally. The pH of the salted and creamed curd needs adjustment. Mealy, pasty, soft, and fragile defects occur in curd made by conventional methods from skimmilk that is overheated while concentrated (e.g., 47% total solids, 160° F. for 15 min.). No detectable changes in whey proteins, A.D.S.A. (pepsin:HCl) curd tension, or solubility index need occur. Rennet curd strength measurements on the reconstituted product and a modification of this test using calcium chloride reflect the severity of the heat treatment given to the concentrated skimmilk.

M57. Determination of the curd-making properties of cottage cheese type nonfat dry milk. W. K. Stone and P. M. Large, Virginia Agricultural Experiment Station, Blacksburg.

Cottage curd was made from an unknown powder in one laboratory vat and from a control powder in a similar vat. The starter and cheesemilk in each vat ewer made from the respective powder being used. Seven pounds of starter were thoroughly mixed with 25.0 lb. of freshly reconstituted 11.0% milk at 95° F., and 0.08 ml. of rennet extract added. When the pH of the curd was 4.70, it was cut into ¾-in. cubes, the whey was increased to 99° F., and at pH 4.60 cooking was started. The curd was cooked at 125° F., washed, drained, packaged, and stored at 40° F. Curd quality was determined by evaluating for body and texture, and color and appearance, using the A.D.S.A. guide for scoring. Comparisons between curd made in small and large vats were made. Preliminary results demonstrate that the correlation coefficients between corresponding small and large vats for curd quality and for per cent moisture were +0.88 and +0.75, respectively; between soluble whey-protein nitrogen of powder and the corresponding curd quality, −0.37, and between rennet curd tension values of powder and the corresponding curd quality, −0.32.

M58. Effects of starters on acidity and firmness of cottage cheese coagulum. D. B. Emmons and W. V. Price, University of Wisconsin, Madison.

One lot of nonfat dry milk was reconstituted (11:89, powder:water) for experiments testing 17 commercial starters. Coagulum was formed at 90° F. with rennet and starter used at rates of 1 ml. and 70 lb., respectively, per 1,000 lb. of skimmilk. There were marked differences in relationships of titratable acidity of whey and pH of curd obtained with the different starters. The range in titratable acidity of whey at pH 5.1 was from 0.45 to 0.66%. High titratable acidities of whey at the A-C endpoint were associated with floating curd. Varied CO_2 production by starters undoubtedly explains differences in titratable acidity of whey at a given pH. Curd strength values measured with the Cherry-Burrell curd tension apparatus were closely correlated to the

decrease in pH after coagulation, regardless of the starter used. The relationship between curd strength and pH of the curd varied with the activity of the starter used; slow starters were associated with higher pH values at the time of coagulation. Starters had a definite effect on the relationship between titratable acidity of whey and curd strength.

M59. Aggregation of calcium caseinate by heat and its reversal. C. A. ZITTLE, Eastern Regional Research Laboratory, Philadelphia, Pennsylvania.

Solutions of calcium caseinate stable at 25° C. precipitate when heated to 90° C. and redissolve at the lower temperature. This reversible aggregation has been studied further by using more dilute $CaCl_2$ solutions, so that the aggregation (turbidity) can be measured in a spectrophotometer. One per cent solutions of casein at pH 6.3 were studied, containing 4 to 6 mm. of $CaCl_2$ per liter. Turbidities were estimated from the light extinction at 600 mμ in a cell at 30°. Solutions were raised from 50 to 90°, held there for various periods of time, then returned to 30°, and the decrease in turbidity followed with time. This aggregation-time reversal curve was compared with the curve obtained when the solution was placed at 30° without prior heating. Whole casein, α-casein, paracasein, and casein with sodium phosphate were studied in detail. The aggregation of whole casein at elevated temperature reversed quickly at 30° and then followed a curve above the 30°-no heat curve, the distance above the 30°-no heat curve was determined by the temperature and duration of heating. Detailed results for this and the other caseins studied will be reported.

M60. Separation of a calcium-soluble fraction of casein from isoelectric casein. K. K. FOX, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

The presence of a calcium-soluble casein fraction in the caseinate complex of milk is of interest, in that its concentration and mode of association with the other constituents of casein may govern the stability of this complex during heating and storage of concentrated milk products. A method for its isolation from isoelectric casein, requiring only equipment normally found in the usual dairy laboratory, is presented. A 2% solution of sodium caseinate at pH 11.0 and 5° C. is made 0.15 *M* with $CaCl_2$. The pH is lowered to 8.3 with *N*/10 HCl and the fraction aggregated by calcium is removed. The supernatant is warmed to 30° C. and passed through a milk clarifier. The clarified liquid is brought to pH 4.7 and the resulting precipitate washed to remove calcium. The precipitate contains two components, the principal component sedimenting at about 11.8 S (Svedberg units) and the lesser component at 1.35 S. The lesser component is soluble, and the main component not soluble, in 6.6 *M* urea. The isolated principal component has a relatively constant S (about 11.8) over the pH range 7.8–9.8, dissociating to exhibit lower values at pH 10.3 and above.

M61. Additional evidence of a stable complex between β-lactoglobulin and α-casein. J. C. TRAUTMAN AND A. M. SWANSON, University of Wisconsin, Madison.

Electrophoretic analyses (phosphate buffer, pH 6.8, ionic strength 0.1) were made on proteins from raw skimmilks, skimmilks that had been heated to 180° F. for 30 min., and skimmilks containing sulfhydryl-blocking agents before receiving a similar heat treatment. In the heated skimmilks, at least two-thirds of the original β-lactoglobulin disappeared, but in the skimmilks containing the —SH blocking compounds the electrophoretic patterns were comparable to the original raw skimmilks. Similar results were obtained with various isolated casein fractions. These results indicate a stable complex and that the —SH groups may be involved in its mechanism.

M62. Electrophoretic and ultracentrifugal analysis of casein complex size fractions from milk. F. A. HECKMAN, W. L. CHOATE, AND T. F. FORD, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Conflicting reports [*(1)* Hostettler, Von H., *et al., Landw. Jarhb. Schweiz,* 63: 31-67 (1949); *(2)* Heyndrickx, G. V., and Vleeschauwer, A. De. *Experientia,* 8: 317-320 (1952)] on the relative concentrations of α- and β-casein in fractions of the caseinate complex separated by differential centrifugation led to a reinvestigation of this system. Fractions were separated and dialyzed against veronal buffer for electrophoretic and ultracentrifugal study. It was found that the ratio of α- to β-casein is essentially constant over a wide range of sizes, in agreement with *(1)*. One component with an S value of 3.5 was predominant in all fractions. The depleted supernatant solutions showed varying ratios of α- to β-casein when prepared for electrophoresis in the same manner, in agreement with *(2)*. It is concluded that the presence of serum proteins in the dialyzed preparation of the supernatant liquid results in changes of interaction between α- and β-casein. The constancy of composition of the centrifugally separated complex is considered to be strong evidence that the particles of various sizes in milk have an essentially constant composition.

M63. Voluminosity of caseinate complex in milk, and reconstituted sediments. C. H. WHITNAH AND W. D. RUTZ, Kansas State College, Manhattan.

Six liquids ranging from 0.444 to 0.162% N were prepared from a sample of milk using *(1)* a commercial cream separator, *(2)* a Sharples supercentrifuge, and *(3)* a Ford "bowl rotor." Sediment from the Sharples bowl was reconstituted with water to contain 0.819% N. Half of this suspension was diluted with an equal weight of water. Such dilutions were repeated until six concentrations of sediment were obtained. Viscosities were measured at 15 temperatures from 4 to 70° C. Fluidities (ϕ) were calculated relative to *(1)* the fraction containing least N, and *(2)* to water. The slope of each series of five or six pairs of values for ϕ and concentration was determined by least squares and converted to voluminosity, using the equation $V = 0.4\ d\ (\phi/\phi)/d$ concentration. Approximate average voluminosities ranged from 6.0 ml/g at 4° C. to 4.8 at 20° C. and 2.8 at 70° C. when ϕ' was the fraction containing least N. Corresponding values when ϕ' was water were 4.5, 3.5, and 2.0 ml/g. Irregularities in plots of V vs. t were larger than can be explained as errors of measurement.

M64. Size memory of casein colloid particles. W. L. Choate, F. A. Heckman, and T. F. Ford, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Fractions of the caseinate complex, prepared by differential centrifugation, followed by dialysis of each fraction against veronal buffer, have been shown to have the same ratio of α- to β-casein by elesctrophoretic analysis. Ultracentrifugal analysis of thees solutions showed one major component with an S value of 3.5 to be present. These preparations when redialyzed against skimmilk become opaque and resemble milk. Ultracentrifugal analysis shows S values in the same range as in the original milk. The fractions with larger S values in milk have larger S values in the redialyzed colloids, ranging from 100 to about 1,000.

M65. Effects of accelerated electron irradiation on the casein of skimmed milk and sodium caseinate sols. J. R. Brunner and R. C. Nickolas, Michigan State University, East Lansing.

The cold irradiation of a casein sol with accelerated electrons changes the multi-component characteristics of the electrophoretic pattern of sodium caseinate to a pattern showing a single component; the μ of which corresponds to the μ of the α-casein component. Ultracentrifugal diagrams of these irradiated caseins show but one sedimentation boundary. These observations are strongly indicative of a complex formation between α- and β-casein. Irradiation levels required to produce the single-peak phenomena are approximately 1×10^6 and 4×10^6 reps for sodium caseinate sol and the casein of skimmed milk, respectively. Other observations showed an increase in the rennet coagulation time, lower curd tension, and lower heat stability at 120° C. as a result of irradiation. No appreciable change in pH or NPN content was noted. Titrations in aqueous, alcohol, and acetone media did not indicate a cleavage either in the peptide linkage or in the phosphobond. Fresh milk plasma dialysate and/or certain of its constituent components exhibited a sparing action on the irradiated casein.

M66. Effects of proteins on agglutination of fat globules. Alan Kenyon and Robert Jenness, University of Minnesota, St. Paul.

Some of the individual components responsible for agglutination of fat globules were investigated. The techniques employed to assess agglutination included visual and microscopic examination, cream layer formation, and fat content of gravity skimmilk. In any one series of tests the source of fat globules was a single lot of cream which was reconstituted with skimmilk and any individual constitutents desired. Either homogenization or heat treatment of skimmilk prevents agglutination of fat globules, but a mixture of equal parts of homogenized and heated skimmilk produces normal creaming, which is in agreement with the report of Samuelsson and coworkers in Sweden. Addition of 15 γ of colostral euglobulin per 100 ml. restored the creaming ability of heated but not that of homogenized skimmilk. On the other hand, β-lactoglobulin, α-lactalbumin, pseudoglobulin, and blood serum albumin, when added to heated skimmilk in the amounts in which they usually occur, had no effect. An attempt was made to identify the factor inactivated by homogenization. Electrophoretic analysis of protein adsorbed on cold fat globules and released upon warming revealed the presence of a component with a motility of -4.5×10^{-5} cm.^{2} volt^{-1} sec^{-1} at pH 8.6, $\mu = 0.1$ in veronal buffer.

M67. A comparative study of the influence of heat, concentration, and rennin action on the sedimentation of proteins in milk. I. S. Verma and C. W. Gehrke, University of Missouri, Columbia.

It was found earlier that the enzymic stage of renning action in milk caused increased association of casein molecules. Further studies were conducted to determine the effect of added Ca- and P-salts. The simultaneous addition of Ca and rennet showed a very significant increase in sedimentation, and the composite effect was more than the additive effects at 8° C., where only the first phase of rennin action occurs, showed an increased sedimentation of N of 64.4 mg. % as compared to the control; whereas, addition of Ca caused an increase of 36.4 mg. % of N. Rennet and Ca addition together increased the sedimentation of N by 173.4 mg. %. The results have been further confirmed by studying milk treated with ion-exchange resins

or oxalate to eliminate the Ca effect. Increased sedimentation of proteins is demonstrated to be the result of establishment of additional intermolecular Ca-bridges on foci created by rennin action on casein. Comparative studies were made on milk concentrated by heat and freeze-drying. The results reveal qualitative similarities but are not significant.

M68. Phospholipid and casein interactions. S. M. HUSAINI AND A. M. SWANSON, University of Wisconsin, Madison.

Complex formation between casein and milk phospholipids during heating of simple systems was studied. Phospholipids were prepared from dry buttermilk solids by extraction with 1:1 ethanol-ethyl ether. Casein was prepared by isoelectric precipitation from fresh skimmilk. In most of the work, solutions were prepared by mixing 3% casein and 1% phospholipids in phosphate buffer (pH 6.8, 0.1 ionic strength) at 3–5° F. These solutions were heated in an oil bath at 180° F. for 15 min. and cooled immediately. The following methods were used as a means of determining complex formation (interaction): nonavailability of part of the phospholipids to ethyl ether extraction, changes in isoelectric point of casein, nephelometry, and viscosity measurements. Using phosphorus determinations as a means of measuring available phospholipids after ethyl ether extraction, it was found that 63.0% of the phosphorus could not be recovered from the casein-phospholipid mixtures after heating. In casein-phospholipid mixtures of very low concentration there was an increase in haze value after heating. A change in the isoelectric point of the casein, and a decrease in viscosity as measured by the Ostwald pipette method, were also observed when the casein-phospholipid mixtures were heated. Similar results were obtained when α-casein was used. These changes would indicate that an interaction took place during heating.

M69. Optical ultracentrifugal resolution of casein complex particles in milk. T. F. FORD, LORRAINE W. KLIPP, G. A. RAMSDELL, AND W. L. CHOATE, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

The air-driven, air-floated capillary tube ultracentrifuge has been used for the study of the casein colloids of whole milk and skimmilk. Various optical techniques have been employed. It has been found that many definite sizes of casein complex particles can be sharply resolved with whole milk, but only a few dominant sizes can be identified with skimmilk. It is suggested that whole milk contains a size-stabilizing substance; that the persistent dominant sizes in skimmilk are determined primarily by physical forces. Sedimentation constants are tabulated.

M70. Application of tritium and carbon^{14}-labeled compounds in cheese ripening. A. WOLIN AND F. V. KOSIKOWSKI, Cornell University, Ithaca, New York.

Milk components were labeled with tritium or carbon,^{14} so as to follow labeled breakdown products of ripening cheese. Randomly tritiated casein was prepared by the Wilzbach technique (*J. Am. Chem. Soc.*, 79: 1013. 1956). Five grams of casein were incubated with tritium gas (H^3) for 72 hr. at 27° C. This tritiated casein which was washed to remove the labile H^3 attained a specific activity of 6 mc. per gram. Due to the low beta energy of tritium (0.0189 Mev), radioautography and windowless–gas flow counting were employed. The windowless counter is more sensitive for C^{14} than the thinnest mica window counter and it also enables the sensing of H^3. A rate of 25 counts per minute was obtained on one sample of labelled C^{14} casein with a thin mica window counter, in contrast to 250 counts per minute with the windowless counter. Labeling of compounds with tritium is less expensive than C^{14}, and a higher specific activity is achieved. The C^{14}-labeled casein displayed 125 c.p.m/0.1 mg., whereas the H^3 casein was 94 c.p.m/6.9×10^{-6} mg. Another advantage is that milk can be directly tagged with tritium, whereas C^{14} must be fed or injected into the cow to label the milk components.

M71. Characteristic Cheddar cheese flavor in relation to hydrogen sulfide and free fatty acids.* T. KRISTOFFERSEN AND I. A. GOULD, The Ohio State University, Columbus.

Fourteen lots of commercial Cheddar cheese were cured under controlled conditions for 1 yr. The cheese was graded and analyzed for hydrogen sulfide and free fatty acids at definite intervals. The rate of development of characteristic Cheddar flavor (C.F.) was greater in raw- than in pasteurized-milk cheese. The C.F. of high-quality, pasteurized-milk Cheddar cheese was considered equal to that of raw-milk cheese after about 6 mo. of curing. Pasteurized-milk cheese, consistently graded B or below, usually was rated lower in C.F. than either the raw-milk cheese or the pasteurized-milk cheese of high quality. Intensity of C.F. appeared to be related to the molar ratios of both 0_5 and longer-chain fatty acids and acetic acid to hydrogen sulfide. Cheese with ratios of $C_5+/H_2S \times 100$ from 1.5 to 5.0 and $C_2/H_2S \times 100$ from 4 to 9 were rated highest in C.F. Ratios outside these ranges were associated with lower C.F. intensities. The concentrations of hydrogen sulfide in the cheese fluctuated during ripening, whereas those of free fatty acids generally showed a continuous increase.

* Supported in part by funds from the American Dairy Association and the Ohio Dairy Products Research Fund.

M72. Identification of volatile carbonyls from Cheddar cheese. E. A. Day and Mark Keeney, University of Maryland, College Park.

Forty pounds of high-quality, rindless, 16-mo.-old Cheddar cheese was macerated, dispersed in water, and distilled at from 25 to 35 mm. Hg pressure. Evolved volatiles were condensed in cold traps, reacted with an acidic 2,4-dinitrophenylhydrazine solution and the 2,4-dinitrophenylhydrazones (2,4-DNPH) were obtained by chloroform extraction. After removal of the chloroform, the 2,4-DNPH mixture was exhaustively extracted with hexane and the hexane extract was condensed and resolved on nitromethane-hexane-Celite partition columns. All solvents were carefully treated prior to use, to avoid artifacts. Identification of the 2,4-DNPH's was based on: *(1)* comparison of threshold volumes of knowns and unknowns on the partition column; *(2)* melting points and mixed melting points; *(3)* absorption spectra in the 220 to 400 mμ region; and *(4)* regeneration of the 2,4-DNPH for characterization of odor properties. The 2,4-DNPH's of 3-methylthiopropanal (methional), formaldehyde, acetaldehyde, acetone, butanone, 2-pentanone, 3-methylbutanal, 2-heptanone, 2-nonanone, acetoin, and diacetyl were identified. The 2,4-DNPH's of 2-tridecanone and 2-undecanone have been tentatively identified by techniques *(1)*, *(3)*, and *(4)* above. Of the carbonyls identified, 3-methylthiopropanal appears to be most noteworthy in Cheddar flavor. A number of the volatile carbonyls have yet to be identified.

M73. Methyl ethyl ketone and other carbonyl compounds in Cheddar cheese. R. Scarpellino and F. V. Kosikowski, Cornell University, Ithaca, N. Y.

Cheddar cheeses at different stages of ripening were obtained from several manufacturers and the flavor intensity of each sample was assigned a place on a numerical scale. Total carbonyl content of the cheeses was determined by colorimetric analysis of the 2,4-dinitrophenylhydrazone derivatives, and the individual carbonyl compounds were separated through paper chromatography. A relationship between flavor intensity and the quantitative and qualitative nature of the carbonyl compounds was tentatively indicated. Among the carbonyl compounds observed were three compounds hitherto unreported in Cheddar cheese chemistry literature. Their chromatographic positions indicated chain lengths of four, six, and seven carbons. The four-carbon compound, identified by its melting point as methyl ethyl ketone, existed at relatively high concentrations in all the aged cheeses. More research is being conducted to establish the origin of these compounds and their contribution to flavor.

M74. Further studies on Cheddar cheese making by the Australian short method. P. A. Downs, University of Nebraska, Lincoln.

Since our first report in 1955, twelve, 300-lb. lots of cheese have been produced by this method, resulting in a slow-curing, good-quality cheese. The slow curing seemed to be related to the lack of acid development in the curd during cheddaring. In an effort to correct this, the originators of the method have recommended the use of *Streptococcus lactis* with *Streptococcus thermophilus* in place of *Streptococcus durans*. This was found to be an improvement. A modified schedule using *S. thermophilus* alone, and suggested later by Australian workers, has been tried without improvement owing to the slow acid development in the curd. The lots of cheese made by the short method over 3 yr. have all cured to a high-quality cheese. The original modified method is considered as a practical, time-saving commercial method of making Cheddar cheese, which tends to cure slowly.

M75. Effect of salt on acid development in Cheddar cheese. H. E. Walter, A. M. Sadler, C. D. Mitchell, and R. E. Hargrove, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Great differences encountered in acid development by single strains of *Streptococcus lactis* and *Streptococcus cremoris* in Cheddar-cheese curd after salting led to this study. For each of 20 experiments, one lot of curd was divided after milling into six equal portions, and varied amounts of salt were added to yield from 1.0 to 2.25% in the cheese. Single strains of *S. lactis* and *S. cremoris,* and mixtures of both species, were used as starters. In general, acid production by most single strains of *S. lactis* was not inhibited by less than 1.6% salt and not significantly by from 1.6 to 2.0%; whereas, most single strains of *S. cremoris* were inhibited slightly by 1.4%, definitely by 1.6%, and almost completely by 2.0%. Some strains of *S. cremoris* were more salt-tolerant than some strains of *S. lactis*. Mixtures of *S. lactis* and *S. cremoris* were much more uniform in salt resistance than single strains of either species. They were not inhibited by less than about 1.5% and not significantly by concentrations between 1.5 and 1.9%. Acid bitterness decreased with increased salt. Fruity flavors usually accompanied insufficient acid development. Cheese containing 2% salt failed to ripen normally.

M76. Acidity and age of natural cheese as factors affecting the body of pasteurized process cheese spread. N. F. Olson, D. G. Vakaleris, W. V. Price, and S. G. Knight, University of Wisconsin, Madison.

Two varieties of cheese, Cheddar and Dariworld, were made with normal and high pH.

They were aged at 50° F. and at various intervals were individually used as the base for cheese spreads of constant composition. The acceptability of the spreads was measured organoleptically, by penetrometer and by melting tests. It was found that natural cheese with high pH was undesirably firm in body at every age, and this firmness persisted in the spreads made from such cheese. All tests showed that the age of the cheese when processed into spread affected acceptability. Improvements were found as the cheese aged to 60 days. After that, penetrometer values tended to show increased hardness and melting tests continued to improve with age. Storage of spreads at 50° F. was possible without significant changes in penetrometer values for at least 90 days, but deterioration was measurable after 15 days of storage at 90° F. and had reached a critical level for commercial use in approximately 30 days. The normal Dariworld cheese appeared to be superior to normal Cheddar cheese for making this type of spread. It reached desirable processing characteristics at an earlier age and the spread made from it was more durable in storage.

M77. Interrelationships between pH, populations of Propionibacterium shermanii, levels of free fatty acids, and the flavor ratings of Swiss cheeses. F. E. KURTZ, J. A. HUPFER, E. A. CORBIN, R. E. HARGROVE, AND H. E. WALTER, Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Published correlations between fatty acid levels and flavor of Swiss cheese have been sufficiently positive to prompt further study. Fifteen experimental cheeses were examined periodically during 12 mo. for fatty acid levels, pH, bacterial population, and flavor; samples of five commercial cheeses were rated on flavor and free fatty acid content only. Variations in pH of the cheeses off the press from 4.95 to 5.37 were experimentally induced. The pH showed a general correlation with the maximum populations of *Propionibacterium shermanii* which ranged, with increasing pH, from 145 million to 2,420 million organisms per gram of cheese. The maximum content of acids ranged as follows: acetic, 38–59; propionic, 50–104; butyric, 6–40, and the higher acids, as a group, 22–48 μM per gram of cheese. There was a correlation between the maxima of propionic acid and *P. shermanii.* The data indicate that the nutty, but not the sweet, flavor may be related to the propionic acid level. It appears that propionic acid is a component of the flavor complex but that other compounds are responsible for both the nutty and the sweet characteristics of typical Swiss cheese flavor.

M78. Continuous, mechanical process for draining and hooping Blue cheese curd. D. M. GRAHAM, Clemson Agricultural College, Clemson, S. C.

A mixture of curd and whey is pumped from the cheese vat into a rotating, cylindrical sieve. After removing the free whey, the curd passes continuously onto an inclined, vibrating chute. A 40-mesh wire sieve mounted on the vibrating chute lightly dusts the curd with dry mold powder immediately before the curd falls into the hoops. Blue cheese curd has been pumped without damage to particle structure by either an Anderson Model 520 or a Waukesha Model DO curd pump at flow rates up to 20 gal. per minute through 1½-in. stainless steel tubing. Some curd damage resulted from use of a standard centrifugal milk pump. The 7½-in. diameter sieve rotated at 20 r.p.m. and was inclined 15 degrees from horizontal. Hoops were filled without hand manipulation of the curd at the rate of two to three per minute. The cheese was set at 90° F. in the usual manner, cut into ½-in. cubes, and pumping started when the whey acidity was 0.16%. Moisture content of four wheels selected at random from a 300-gal. vat ranged from 44.47 to 45.24% and fat content ranged from 30.0 to 30.5%. Mold scores after 3 wk. of aging averaged one to two points higher for machine-hooped than for manual-hooped cheese. Distribution of veins was markedly improved, probably owing to a more uniform inoculation and open texture in the machine-hooped cheese.

M79. Selection of Penicillium strains for Blue cheese. D. M. GRAHAM, Clemson Agricultural College, Clemson, S. C.

Eight strains of *Penicillium,* three from laboratory collections and five isolated from cheese, were studied. Criteria for evaluating the strains were *(1)* organoleptic scores of cheese inoculated with each mold, *(2)* volatile acidity, per cent nonprotein nitrogen, and neutral carbonyl compounds in the cheese, and *(3)* ability to produce neutral carbonyl compounds in laboratory cultures. Statistical treatment of the organoleptic scores of 14 vats of cheese ranked the strains in five groups: *(1)* P12, P108, M; *(2)* R, *(3)* I, C; *(4)* B, and *(5)* N. Under ideal curing conditions, strains M and R produced excellent flavor, but in routine manufacture frequently they failed to develop adequate veining. Addition of 0.2 to 0.4 γ $FeCl_3$ per 100 g. milk did not improve growth of strains M and R. Strains I, P12, and P108 consistently produced well-veined and good-flavored cheese in routine manufacture, under a variety of conditions in natural cave and mechanically refrigerated curing. Inoculation with two, three, or four strains in 15 different combinations produced less desirable cheese than single-strain inoculation. Correlation between flavor scores, volatile acidity, and neutral carbonyl compounds was fair.

M80. Development of a domestic Feta cheese. C. EFTHYMIOU AND J. F. MATTICK, University of Maryland, College Park.

Cheeses were made from pasteurized and raw milk. The lipolytic agents included in the study were natural milk lipase and Dairyland Food Laboratories' "pregastric-esterases"—Capalases K, L, KL, and Italase C. The bacterial cultures used, pure and in mixtures, were *Streptococcus lactis, Lactobacillus acidophilus,* and *Lactobacillus casei.* The milk was ripened with 1% starter at 86° F. until 0.025% acid developed. Commercial lipases were added at a rate of 1.5 g/100 lb. milk. Milk was coagulated with liquid rennet at 86° F. in from 40 to 60 min. Curd was cut with 1-in. knives, stirred with mild agitation at 86° F. for 20 min., then dipped into perforated 12-in.-cube cheese forms. After sufficient drainage, the curd was removed from the forms, cut into 4-in. squares, and placed in a curing room at 65° F. and 85% relative humidity. The cheeses were dry-salted at a rate of 3% of the weight of the cheese. After from six to eight days of curing, the cheeses were washed, placed in sealed cans with sufficient 3% NaCl solution to fill the cans, and stored at 40° F. Feta cheese with excellent flavor and body and texture characteristics was developed using pasteurized milk, Capalase KL, and a mixed culture of *S. lactis* and *L. casei.*

M81. The vitamin B_6 potency of cheese. I. L. HATHAWAY, University of Nebraska, Lincoln.

The vitamin B_6 potency of 406 samples of 31 cheeses was determined by microbiological methods. The cheeses included in this study were American, Beer Kaese, Blue, Brick, Camembert, Caraway, Wisconsin Cheddar, New York Cheddar, Cheese spread, Cottage, Dry Curd Cottage, Cream Cheese, Philadelphia Cream, Edam, Garlic, Gouda, Gruyère, Kaukauna Klub, Liederkranz, Limburger, Mozzarella, Muenster, Parmesan, Pepper and Bacon Cheese, Port Salut, Provolone, Roquefort, Swiss, and Velveeta. These samples were obtained from the Nebraska Experiment Station Creamery, from cheese factories and stores in Lincoln and Omaha, Nebraska, or from Milwaukee, Wisconsin. Since the samples were representative of the cheeses which are available in most markets, the results are believed to indicate the vitamin B_6 value of this product. The B_6 values ranged from 44 γ per 100 g. of dry curd cottage cheese (19 samples) to 251 γ per 100 g. of Camembert cheese (21 samples). Of the 31 cheeses studied, Camembert, Liederkranz, and Blue cheese were the most potent in vitamin B_6.

PRODUCTION SECTION

P1. Formulation and feeding of purified diets to dairy cattle. J. H. BYERS AND J. R. STAUBUS, University of Illinois, Urbana.

Sixteen steers and one heifer were fed various purified diets and performance was measured by body weight and digestion trials. Gains followed consumption closely. Diet A containing 55% Solka-Floc, 22.8% corn starch, 10% cane sugar, 4% urea, 4% hydrogenated tallow, 4% minerals, and 0.2% A and D supplement was very unpalatable. Reducing Solka-Floc to 35% and increasing sugar to 14.8% and corn starch to 38% increased palatability and fiber digestibility. Observations and digestion trials showed that the tallow was not well digested. When corn oil replaced tallow, palatability and crude fiber digestibility were increased. The A and D supplement was replaced by purified fat-soluble vitamins without observable effect. Water-soluble vitamin supplementation individually or as a complex failed to improve gains. Inadequacies in the diets were indicated by impaired and depraved appetites, loss of condition, malfunction of joints, impaired vision, and subclinical infections at terminal slaughter. Four animals died of unknown causes. One animal showed symptoms of urea toxicity. Replacing urea with Drackett improved gains but not digestibility. Ten per cent ground cobs increased palatability, gains, and crude fiber digestibility. Work is continuing to define this effect of cobs.

P2. Effect of all-hay vs. all-silage rations on dry matter intake of lactating dairy cows; moisture and pH as factors affecting appetite. D. HILLMAN, C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, Michigan State University, East Lansing.

Two equalized groups of eight milking Holstein cows were used in a 42-day single-reversal experiment to determine the effects of an all-hay ration compared to an all-silage ration on dry matter intake, body weight, and milk production. The hay and wilted silage (25.0% D.M.) were both harvested from the same field at the same stage of maturity. Cows fed all hay averaged 3.14 lb. of dry matter per 100 lb. of body weight, gained 19.4 lb. in body weight, and averaged 28.3 lb. of milk per day. Respective values for cows fed silage were 2.46, −42.4, and 28.4. Intake and body weight changes were significantly different (1%). In a 40-day Latin-square design, trial cows were fed *(1)* regular hay, *(2)* regular silage, *(3)* hay soaked with water, 30.6% D.M., and *(4)* high pH silage, regular silage sprayed with NaOH to increase the pH to that of the hay. Milk production, gain in body weight, and dry matter intake values for regular hay were 23.4, 10.7, and 3.23; regular silage—21.6, −36.9, and 2.17; wet hay—22.4, 15.0, and 3.28, and high pH silage—21.2, −22.6, and 2.21. Differences among rations in dry matter intake and body weight changes were significant in both hay groups as compared to the silage groups, but not within each roughage.

P3. Effect of various additives on dry matter intake of heifers fed either alfalfa hay or sil-

age. J. P. EVERETT, JR., C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, Michigan State University, East Lansing.

Twenty-four heifers averaging 674 lb. body weight were divided into three groups and offered either all hay (Group 1), wilted silage averaging 25.0% D.M. (Group 2), or lacerated-high-moisture silage averaging 17.9% D.M. (Group 3), free choice in a 4-wk. trial. All three forages were harvested from alternate areas of the same field of alfalfa in one-quarter bloom. The silages were stacked on a concrete slab with no preservative and covered with a plastic sheet. In a second trial, each group was subdivided and half of the animals fed the following additives: hay sprinkled with dilute HCl, wilted silage plus a flavor compound (Silo-joy), or high-moisture silage plus ground hay. Average daily gain and average daily dry matter intakes per 100 lb. body weight for Groups 1 through 3 were 1.69, 1.28, 0.09 lb., and 2.33, 2.01, and 1.60 lb., respectively, for Trial 1. Lowering the pH of the hay and adding Silo-joy to wilted silage had no significant effect on the dry matter intake in Trial 2. However, adding enough ground hay to the high-moisture silage to raise the dry matter content to approximately that of the wilted silage resulted in a significant ($P < 0.05$) increase in dry matter intake.

P4. Studies on forage: concentrate ratios for milk production. J. L. MORRIS, E. GAINER, AND R. F. DAVIS, University of Maryland, College Park, AND C. R. RICHARDS, University of Delaware, Newark.

Six trios of dairy cows were assigned to the following rations starting 20 days prepartum and continuing throughout a 305-day lactation: *(1)* 80% of the estimated net energy (Morrison: upper level) from concentrates, 20% from hay, *(2)* 40% of the estimated net energy from concentrates, 60% from hay; *(3)* 20% of the estimated net energy from concentrates, 80% from hay. Following parturition all animals were fed ad libitum up to 110% of Morrison's recommended allowances. Beginning with the 6th wk. and until the end of lactation, the estimated net energy intake was lowered 1% of the intake of the previous week for all treatments. U. S. No. 2 leafy alfalfa and a simple grain mixture were fed throughout the experiment. Observations on the incidence of mastitis and udder edema were made periodically throughout the experiment. Digestion studies on the above rations were conducted with a change-over design with three steers. In addition, the chromic oxide indicator method was used to determine the digestibility of the rations on 13 cows. The production of 4% fat-corrected milk averaged 30.9, 27.2, and 25.1 lb. per day, with the rations ranked in decreasing proportion of concentrate. After allowing for maintenance and body weight changes, the calculated therms required per lb. of 4% F.C.M. were 0.40, 0.35, and 0.34, in the same order.

P5. Effect of level of feed intake using hay: grain ratios on feed utilization of dairy cows. C. A. LASSITER, C. F. HUFFMAN, AND C. W. DUNCAN, Michigan State University, East Lansing.

In a previous study, using good alfalfa hay, it was observed that as the level of feed intake increased the digestibility of hay: grain rations increased. In this study, 27 digestion trials were conducted, using nine dry cows to study this phenomenon further. A medium-grade, late-cut timothy hay and a 21.2% protein-grain mix were fed. Three hay-grain ratios were fed, 80: 20, 50: 50, and 20: 80, on a dry matter basis. Three levels of intake were used—70, 100, and 130%, where the 100% level was a typical rate of feeding dry cows averaging 23.7 lb. of feed per day. The trend toward higher coefficients of digestibility, with an increasing proportion of grain to hay, was evident with all nutrients except crude fiber. There was a definite trend toward higher digestion coefficients as the level of intake increased, although not statistically significant, except with crude protein and crude fiber. This was evident at all hay: grain ratios. The average coefficients of digestibility, regardless of hay: grain ratio, for the high, medium, and low levels of feed intake were 75.4, 70.7, and 69.4 for dry matter, 77.7, 75.4 and 71.1 for crude protein, 62.2, 50.8, and 46.4 for crude fiber, and 76.6, 72.2, and 70.3% for organic matter, respectively. This trend was most pronounced with crude fiber digestion and with the 20: 80 hay: grain ratio.

P6. Effect of supplemental fat in high-energy rations on hot-weather performance of producing dairy cows. R. R. HARRIS, R. E. LEIGHTON, AND I. W. RUPEL, Texas A & M College, College Station.

The energy value of the low-fiber rations used in hot-weather studies (*J. Dairy Sci.*, 40: 1391. 1957.) was increased further by increasing the fat content in the concentrates from 3.8 to 8.9%. Fat additives used were tallow and a cottonseed "foots" methyl ester product (Marcol B-75). During the summer of 1957, these high-fat, low-fiber rations were compared with the same high-fiber control ration used in previous studies, when fiber was the only variable. Because the estimated net energy supplied in each ration was kept to 105% of the cow's requirements, 21% less feed containing a total of 37% less fiber, was fed in the high-energy ration as compared to the control. The differences in favor of the high-fat, low-fiber ration were: milk, +2.1 lb. per cow per day; body temperature, −0.7° F.; respi-

ration rate, −3.6, and pulse rate, −2.7 per minute. The corresponding figures when the low-fat, low-fiber ration was compared with the high-fiber control ration averaged: +1.6 lb., −0.3° F., −6.4, and −4.0, respectively. The comparatively small differences in average daily milk production in favor of the high-energy ration for the 84-day summer trials were much less than the differences observed during the hottest weather.

P7. Effect of feeding tetraalkylammonium stearate to milking cows. A. C. LINNERUD, J. D. DONKER, AND J. B. WILLIAMS, University of Minnesota, St. Paul.

Ten cows, past peak milk flow and not more than 59 days pregnant, were paired and randomly allotted to control and experimental groups. A double-reversal feeding trial (28-day observation and 7-day change-over periods) was used. Chopped alfalfa-brome hay to satisfy appetite, and concentrate to balance nutrient intake, according to Morrison's Standard, were fed. Concentrate feeding was adjusted for each animal each period, to provide nutrients slightly above requirements. Tetraalkylammonium stearate (TAS) in a bone meal carrier (Dynafac) was included in the ration at 300 mg. TAS/cow/day. Daily milk weights and feed intakes were recorded. Weight changes were based on three-day weights at start and finish of each observation period. A two-day composite milk sample was analyzed each week for fat. Daily hay and bi-weekly concentrate samples were composited by periods for analysis by A.O.A.C. methods. Control values are followed by experimental values on a per-cow-per-day basis: milk production (4.0 fat corrected), 18.1 vs. 17.8 lb., concentrates consumed—6.8 vs. 7.0 lb., hay 23.0 vs. 23.2 lb., weight gained, 0.41 vs. 0.38 lb.

P8. Effect of certain measured environmental influences on the butterfat yield of Holstein-Friesian cattle.* E. E. STARKEY,** E. L. CORLEY, AND E. E. HEIZER, University of Wisconsin, Madison.

Environmental and butterfat production records were studied, using herd data collected for 3 yr. from 43 Holstein-Friesian herds in southern Wisconsin. Data were obtained monthly from DHIA field supervisors and through quarterly farm visits. Variables studied were: *(1)* butterfat yield (2×, 305-day, M.E.), *(2)* lb. of TDN fed daily, *(3)* lb. of protein fed daily, *(4)* nutritive ratio, *(5)* length of preceding dry period, *(6)* condition at freshening, *(7)* days carried calf, *(8)* calving interval, *(9)* herd size, *(10)* summer feeding, *(11)* housing, *(12)* milking practice, and *(13)* feeding practice. Variables 1–8 were recorded on each cow, whereas variables 9–13 were on a herd basis. The influence of Variables 2–13 on butterfat yield was estimated by multiple correlation techniques with 1,168 production and environmental records. Over-all herds, net partial regression coefficients of butterfat yield on Variables 2, 5, 7, 8, 9, 11, 12, and 13 were highly significant. Variables 2 and 13 were highly significant on a within-herd basis. Net partial regressions of butterfat yield on Variables 2 and 13 were 13.50 and 0.63 over all herds, and 23.31 and 0.49 within herds. Variation in butterfat yield associated with Variable 2 was 15% within herds, and 10% over all herds.

* USDA support.

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P9. Effect of feeding thyroprotein to dairy heifers. V. SINGH AND J. D. DONKER, University of Minnesota, St. Paul.

Five sets of identical-twin Holstein heifers, about 15 mo. old, were used. One member of each set received the control hay: grain ration, in which an alfalfa-grass hay was fed free choice and the grain was limited to 2 lb. daily and the other member of each set received thyroprotein in its grain. During a preliminary period, it was ascertained that from 7 to 7.4 g. per animal per day was required to increase pulse rate approximately 10% above normal, a level arbitrarily selected for comparison. The following data were gathered during a 6 wk. comparison period: heart rate (beats per minute), six-day average weights, weight gained—absolute gain (lb. per day) and relative gain (% increase per day), feed consumed—absolute quantities and lb. per cwt. The average values for the control data are followed by average experimental values: heart rate—89.3 vs. 96.6; average weight—666 vs. 651 lb.; weight gained—1.37 vs. 1.35 lb. per day and 0.22% vs. 0.22% increase per day; feed consumed—2 vs. 2 lb. grain daily and 16.8 vs. 16.4 lb. hay daily, and total feed per cwt. per day 2.52 vs. 2.52 lb.

P10. Methods of adjusting roughage intake data of dairy cows for differences in body size. R. E. MATHER AND A. A. RIMM, New Jersey Agricultural Experiment Station, Sussex.

Comparing roughage intake of cows of different sizes may lead to different conclusions, depending on the method of adjustment. Use of the ratio of intake to body weight (W) may overcorrect the data. Legates *et al.* proposed the use of regression for adjustment. Data were used from a study in which excellent alfalfa hay was fed ad libitum to cows of four breeds in 38 lactations, with limited corn silage and no grain, or grain at 1 lb. per 6 lb. FCM, or grain at 1:3. Regression adjustment provided a better fit than ratio adjustment, particularly for cows on the no-grain

ration, reducing the sum of squares of the unadjusted values by 66% compared to 24%. Limitations of data sometimes may prevent the adjustment by regression. Adjustment by ratio of intake to W^{73} gave adjusted values close to the regression-adjusted values on all three rations. These two methods ranked cows within ration the same in 26 of 38 cases. The reductions in sums of squares on the three rations were, respectively, 60, 48, and 71%, compared with 66, 56, and 71% by regression.

P11. Comparison of feeding corn cobs and a liquid urea-molasses-mineral mix with and without ethyl alcohol to yearling dairy heifers.* J. P. BATES, JR., D. R. JACOBSON, J. W. RUST, AND D. M. SEATH, University of Kentucky, Lexington.

Twenty dairy heifers, ten Holsteins and ten Jerseys, were divided into two equal groups and were individually fed ad libitum but separately a ration of ground corn cobs and a liquid urea-molasses mix with and without ethyl alcohol for a 168-day trial. Group M received the mix with ethyl alcohol, and group A the mix without ethyl alcohol. The average daily gains for 140 days, following a 28-day preliminary period, were 0.73 and 0.80 lb. for Groups M and A, respectively. This difference was not statistically significant. Groups M and A consumed 14.3 and 15.0 lb. of corn cobs and 5.5 and 4.8 lb. of liquid mix per animal per day, respectively. In the same order, pounds of feed per pound of grain were 27.1 and 24.8. Similarly, water consumption per 1,000 lb. body weight per day was 61.3 and 61.1 lb. when offered water continuously, and 56.7 and 54.4 lb. when watered once a day. For Groups M and A the average blood urea values were 17.27 and 14.59 mg. % and the plasma protein values were 5.86 and 5.78 g. %. Rumen fatty acid percentages were acetic, 68 and 67, propionic, 17 and 18, butyric, 10 and 10, valeric, 4 and 3, and the higher acid fraction, 0.9 and 0.9.

* This work was supported in part by Feed Service Corp., Crete, Nebraska.

P12. A proposed method for the measurement of the respiratory exchange and energy expenditure of grazing animals. W. P. FLATT, D. R. WALDO, AND J. F. SYKES, Nutrition and Physiology Section, USDA, Beltsville, Maryland.

Large-animal energy metabolism studies have required the use of direct calorimeters, respiration chambers, closed-circuit spirometers, or long-term feeding trials in combination with body composition data. These methods are unsuitable for determining the energy expenditures of animals on pasture. A procedure has been devised to determine the O_2 consumption, CO_2 production, and energy expenditure of large animals for extended periods of time without restricting or confining them. The method involves the use of a light-weight (3.6 Kg) portable dry gas meter (Müeller-Franz Gas Meter, 100 L/min capacity, 1.5-in. intake outlet) with an aliquoting device, a 100 L. Douglas bag for collecting a continuous sample of expired air, harness, respiratory valve, and an animal fitted with a tracheal cannula. The total volume of expired air is measured by the meter, which is connected to the tracheal cannula and 0.1 or 0.2% of the expired air is continuously removed and collected in the Douglas bag for subsequent analysis for CO_2 and O_2. Preliminary studies have indicated that this method may be used for determining the energy expenditure for periods of approximately 12 hr. Longer experimental periods are made possible easily by changing the Douglas bags.

P13. Digestibility of orchard grass hays as influenced by two methods of nitrogen application.* R. A. MARKLEY, J. L. CASON, AND B. R. BAUMGARDT, New Jersey Agricultural Experiment Station, New Brunswick.

Four 7-mo.-old dairy heifers were used in a total collection digestion trial, ten-day preliminary and seven-day collection periods, employing a Latin-square design balanced for residual effects, with an extra period added to more effectively estimate carry-over effects. The four rations, fed twice daily, were second-cutting alfalfa and three first-cutting orchard grass hays; *(1)* low—0 lb. N/A, *(2)* low plus urea to equal protein content of the high, and *(3)* high—200 lb. N/A. A nitrogen balance was determined for each ration. The crude protein and digestion coefficients of protein for alfalfa were 16.45 and 71.41%; orchard grass hays—low, 6.47% and 44.99%; low plus urea, 15.82% protein equivalent and 77.77%; high, 15.07 and 70.72%, respectively. Ether extract and gross energy followed a trend similar to protein; whereas, the per cent of fiber and NFE decreased with increasing nitrogen fertilization. The digestion coefficients of protein, NFE, ether extract, and energy increased, whereas that of crude fiber decreased slightly with increasing nitrogen fertilization. The TDN and digestible energy (cal/g) of alfalfa were 49.04% and 2414; low, 47.46% and 2159; low plus urea, 55.44% and 2158, and high, hay 51.59% and 2511, respectively. The statistical analysis will be presented.

* Supported in part by a grant from E. I. duPont de Nemours and Co.

P14. Effect of high ash materials on use of the fecal chromogens method of determining digestibility. C. R. RICHARDS AND W. G. CROTHERS, University of Delaware, Newark.

The ash content of lima bean vine silage is very variable, owing to the method of harvest. With high-ash lots of silage, poor agreement between the chromogens method and conven-

tional or chromium sesquioxide methods of determining digestible dry matter has been noted. With 10.6% ash, digestible dry matter was 53.3 and 53.3%; at 19.2% ash, 53.3 and 48.5%; at 30.9% ash, 61.6 and 36.1% for the fecal chromogens and other methods, respectively. When the fecal chromogens were calculated to an organic matter base and digestion coefficients determined for the organic matter, the agreement between methods was much more satisfactory: 54.8 and 57.4; 57.2 and 57.8, and 53.5 and 54.7, respectively.

P15. Trace-mineral supplementation for dairy cows. E. G. Moody, J. M. Conover, and A. P. Cook, Arizona State College, Tempe.

Using ten pairs of Holstein-Friesian cows in a 22-wk. reversal trial, no effect could be attributed to trace-mineral supplementation of a ration of high-quality alfalfa hay, simple grain mix, bone meal, and salt from measurements of milk production, body weight changes, general condition, hemoglobin, hematocrit, and in the serum: sodium, potassium, calcium, phosphorus, magnesium, chlorine, carotene, and vitamin A. All values, with a few individual exception, were within, or above normal published values. Cows preferred fortified to unfortified salt about 2:1, when given free access to both.

P16. Magnesium sulfate, grain, or alfalfa hay as sources of magnesium to calves fed whole milk. J. W. Thomas and M. Okamoto, Nutrition and Physiology Section, USDA, Beltsville, Maryland.

Whole milk at the rate of 10 to 12% of body weight was fed to 12 calves. When serum magnesium concentration became subnormal (48–131 days of age), magnesium in amounts equal to that supplied by the milk was added as magnesium sulfate, grain, or alfalfa hay. Serum magnesium concentrations gradually decreased as the experiment progressed. The extent of this decrease from the fourth to the tenth month was 1.16, 0.48, and 0.45 mg. magnesium per 100 ml. serum for the $MgSO_4$, grain- and hay-supplemented calves, respectively. Magnesium concentration in ash of rib-biopsy sections taken at 240 days of age was 0.66, 0.67, and 0.75%, respectively, for the $MgSO_4$, grain- and hay-supplemented calves. Magnesium in the ash of rib ends at death (280–467 days of age) was 0.40, 0.54, and 0.69%, respectively. A dietary intake of magnesium of 0.8 to 0.9 gm/cwt was inadequate when half the magnesium was supplied by whole milk and half by either $MgSO_4$, grain, or hay. In this trial, the magnesium in grain and hay was apparently utilized better than magnesium in $MgSO_4$, but the differences were not statistically significant.

P17. Determination of methoxyl in the presence of carbohydrates. W. C. Jacobson and H. G. Wiseman, Nutrition and Physiology Section, USDA, Beltsville, Maryland.

A modification of the apparatus designed by Clark has been used for the determination of methoxyl in the presence of carbohydrates. Modifications include a glass bubbler for sweeping the CO_2 through the hydriodic acid, and a coarse-fritted glass bubbling device for passing the gas through the acetic acid-bromine-potassium acetate solution in the receiver. The trap contained a 1% starch solution to hold any free iodine. Distillations were carried out at steam-bath temperatures. It has been demonstrated that both Bureau Standards dextrose and α-cellulose give a methoxyl value at a linear rate for at least 8 hr. Corrections are made for the presence of carbohydrates by making an additional collection of equal duration and subtracting this value from the first run. Ninety minutes are required to give quantitative recovery of vanillin in the presence of 0.5 g. cellulose, when the value of the second 90-min. collection is subtracted as a blank. When lignin in hay is estimated from methoxyl values, the error due to carbohydrates must be considered.

P18. Determination of sugars in silage with micro-Somogyi copper reagent. H. G. Wiseman, J. C. Mallack, and W. C. Jacobson, Nutrition and Physiology Section, USDA, Beltsville, Maryland.

An improved procedure has been devised for the determination of sugars in silage. The extractions are made in a Waring Blendor, with 80% ethyl alcohol. The extracts are filtered through a bed of Darco G-60 and Celite 535, to give practically colorless solutions. Aliquots are stirred with a mixture of ion-exchange resins, Duolite A-4, and Dowex-50. The deionized aliquots are dealcoholated on the steam bath, made to 1- normal with hydrochloric acid, and allowed to stand overnight to invert. The inverted solutions are neutralized, made to volume, and 10-ml. aliquots heated with 10 ml. of Somogyi's copper reagent (1945). After cooling, the contents are titrated with thiosulfate, stirring being conveniently accomplished with a magnetic stirring bar. Since the sugar solutions have been clarified before bringing nearly to dryness to dealcoholate, economy of volume size is possible; troublesome solution and filtration of gum residues are avoided, and duplication improved.

P19. Effect of adding water to the concentrate mix on maximum milking rate, average milking rate, and eating rate. E. W. Hupp and R. C. Lewis, Michigan State University, East Lansing.

The maximum rate of milk flow, average milking rate, and concentrate eating rate were determined for 16 Brown Swiss cows in loose-housing for six consecutive milkings. Following these observations, water was added to the concentrate mix at the rate of 1.5 lb. per pound of

feed. After a three-day adjustment period, the effect on the above characteristics was determined for six additional milkings. The average maximum rate of milk flow was 3.73 lb. per minute on dry feed and 3.60 lb. after water had been added. The average rate of milking was 2.19 lb. per minute on dry feed and 1.92 lb. per minute on wet feed. Neither of these differences was significant. Eating rate increased significantly ($P = >0.01$) when water was added to the concentrate mix. On dry mix the cows ate 0.63 lb. per minute, whereas the rate increased to 0.90 lb. with the addition of water. Five days later the rates were determined for two milkings. The maximum milking rate was 3.83 lb., the average rate 2.0 lb., and the average eating rate, 0.56 lb. per minute. These results support reports that the concentrate eating rate can be increased by the addition of water. No evidence of an effect on milk flow was found.

P20. Effect of some weather factors on daily milk and fat production. F. N. DICKINSON, USDA, AND H. W. NORTON AND R. W. TOUCHBERRY, University of Illinois, Urbana.

One hundred and one dairy cows from a single herd, including purebred Holsteins and Guernseys and Holstein-Guernsey crossbreds, were sampled for A.M. and P.M. milk yield and composite butterfat test, at ten-day intervals from May 1 to September 30, for 5 yr. The amount of fat produced each sampling day was calculated. The maximum, minimum, and mean temperature, 1 P.M. relative humidity, and mean wind-speed for each sampling day were taken from local weather bureau records. An individual quadratic curve was fitted separately to each type of data for each cow. The deviations from each estimated curve were calculated and summed over cows within dates for the same summer. These within-date sums of deviations from fitted quadratics were analyzed by the method of multiple regression, to ascertain the effect of maximum, minimum, and mean temperature, relative humidity, and mean wind-speed on milk and fat production.

P21. A study of milk flavors. C. F. FOREMAN, E. W. BIRD, F. E. NELSON, W. S. ROSENBERGER, AND M. R. LAMBERT, Iowa State College, Ames.

The milk from each of 169 cows from five breeds in the College herd was tasted monthly during 1957, to establish flavor characteristics and patterns. Three experienced judges independently gave numerical flavor scores (40 perfect) and listed the type of flavor for each sample. Marked differences in flavor and flavor scores existed among animals under the same managemental conditions. The mean score for all cows varied from 34.7 in January to 35.9 in June, September, November, and December. Feed was the most common flavor noted and since normal feed flavor scores varied over a fairly wide range, it suggests a need for study and cataloging of undesirable and acceptable feed flavors and flavor intensities. Common flavor defects other than feed were cowy, oxidized, flat, unclean, and salty. Cowy flavors occurred more often during the winter. Oxidized type flavors were not confined to winter, although the defect was more pronounced during this period. These flavors occurred most frequently during early lactation, although some cows showed them throughout a lactation. The milk from some cows characteristically contained certain flavor defects. The incidence of rancidity was quite low. Further data on flavor patterns will be presented.

P22. Effect of thyroprotein on metabolic and productive function of lactating Jersey cattle exposed to hot and cool weather. J. E. JOHNSTON, G. A. HINDERY, G. T. SCHRADER, AND F. B. HAMBLIN, Louisiana State University, Baton Rouge.

Twenty lactating Jersey cows were divided into four groups on the basis of milk production. Two groups were placed in the climatic control chamber and two groups in an open shed. Conditions in the chamber were adjusted to simulate subtropical summer weather. Conditions in the open shed generally ranged between 40 and 65° F. One group of animals in each location was fed 1½ g. of thyroprotein per 100 lb. body weight. All animals were fed 3 lb. of grain in addition to 1 lb. per 4 lb. of FCM and roughage ad libitum. Thyroprotein feeding resulted in increases in heat production, body temperature, respiratory activity, milk production, and fat percentage. During 40 days under hot conditions, control animaals lost an average of 55 lb. each, whereas thyroprotein animals lost an average of 136 lb. Under cool conditions, controls gained 5 lb. each, whereas thyroprotein animals lost 25 lb. Under hot conditions, controls declined in milk production initially, whereas thyroprotein groups maintained production for the first 20 days then declined, showing severe distress associated with high body temperatures, decline in roughage consumption, and loss of weight.

P23. Effects of high levels of vitamin D_2 fed prepartum on the incidence of parturient paresis in dairy cattle. J. C. DELL AND B. R. POULTON, University of Maine, Orono.

Studies were begun in 1956 to determine the value of high-potency irradiated ergosterol, about seven million U.S.P. units per gram, as a preventive for parturient paresis in dairy cattle. Thirty-one herds of Jersey cows are being used. All cows calving for the third time or more are assigned to four experimental groups. Groups I and IV include only animals with a previous history for parturient paresis. Groups II and III include only animals with no previous history for parturient paresis. Groups I and II serve as treatment groups, whereas

Groups III and IV are controls. The preventive feeding program used is as follows: Beginning approximately 72 hr. prepartum and continuing until 48 hr. postpartum, all animals in the treatment groups received 30 million U.S.P. units of vitamin D_2 daily in their grain. Thus far, data have been collected for over 400 parturitions. The percentage incidence of clinical cases of parturient paresis (requiring calcium therapy) has been as follows: Group I, 32.15%; Group II, 9.15%; Group III, 19.69%, and Group IV, 65.38%.

P24. A calcium lactate–aluminum hydroxide preparation as a milk fever preventive. G. M. WARD, Colorado State University, Fort Collins.

A commercial product* containing calcium lactate and aluminum hydroxide, with and without vitamin D_3 to supply about 500 units per day, was fed to dairy cows for an estimated eight days prior to parturition. This product supplied about 20 g. of calcium per day. Seventeen cows in the University herd with a previous history of milk fever received this material by capsule or drench, and blood samples were analyzed for calcium and phosphorus. Five of these cows developed milk fever and three suffered relapses. Serum calcium and phosphorus levels were no different than those which have been reported previously for cows under these conditions. All three cows which received the material for longer than 11 days developed milk fever. Thirty-two cows in private herds with a previous history of milk fever were fed the product in their grain. Three of these cows were treated for milk fever. Additional vitamin D had no advantage over the product without this vitamin.

* The product Paracalein and financial support was furnished by Warner-Chilcott Laboratories, Morris Plains, New Jersey.

P25. Production of polyvalent antibodies in milk absorption and prophylactic and therapeutic value in calves. MOHAMMAD SARWAR, W. E. PETERSEN, AND BERRY CAMPBELL, University of Minnesota, St. Paul.

Polyvalent-killed antigen were prepared from the cultures of *Pasteurella, Escherichia coli, Streptococci, Corynebacterium, Salmonella enteritidis, Staphylococcus albus* and *aureus,* and *Aerobacter aerogenes,* and were infused intramammarily in 18 cows with antibody production against each organism. The polyvalent antibody milk was fed to eight calves from 2 to 5 mo. of age and, in two to three days of feeding, specific antibodies were demonstrated in the blood. In test for the prophylactic value of the antibody so produced, the polyvalent antibody milk was given to five experimental calves for seven days before beieng challenged with 60 billion cells of pathogenic *E. coli* per ovum; whereas, the control calves continued on the normal milk. The five experimental calves survived and the controls died. Test for the therapeutic value of gamma globulin prepared from polyvalent antibody milk when it was intravenously injected (0.5 grain per 100 lb. of body weight) for three days at the height of the disease, resulted in prompt recovery of all five calves. Two controls died and two survived with prolonged illness and acute arthritis.

P26. Antitryptic factor and antibody absorption. W. E. PETERSEN, MOHAMMAD SARWAR, AND BERRY CAMPBELL, University of Minnesota, St. Paul.

Experiments based on the antitryptic factor in soybean flour were set up to test the validity of the concept of antibody absorption from the G.I. tract in adult animals. Antibody absorption occurred because the proteolytic enzymes are overwhelmed, permitting unaltered, immune protein to enter the small gut. Twenty-eight adult rabbits were used, in which varying levels of toasted soybean flour (antitryptic factor destroyed) for the controls and raw soybean flour for the experimentals were used, admixed with the polyvalent antibody milk fed ad libitum. Levels of the soybean flour varied from 1 to 20% by weight of the milk. The polyvalent antibody milk was prepared from antigen consisting of equal numbers of *Escherichia coli, Salmonella enteritidis, Streptococcus hemolyticus, Staphylococcus albus, Staphylococcus aureus, Corynebacterium,* and *Pasteurella bovisepticus.* Only antibodies against *E. coli* and *S. enteritidis* were titered. The milk agglutinated at 1 to 1,000 dilution. All rabbit sera were negative before the experiment began. All animals on antibody milk acquired antibodies in their sera. Those on milk with the antitryptic factor picked up sera antibodies at five- to more than tenfold the levels in those on the heated soybean flour, where the antitryptic factor was destroyed.

P27. Milk residues associated with use of experimental animal systemic insecticides. J. E. CASIDA, R. P. NIEDERMEIER, J. B. KNAAK, W. C. DAUTERMAN, H. R. KRUEGER, AND F. W. PLAPP, University of Wisconsin, Madison.

Lactating cows were treated with three experimental animal systemic insecticides containing radioactive phosphorus. One to three cows were used per compond. The rate of secretion of the insecticides and/or their metabolites in the milk was studied. Materials studied were: Trolene or ET-57 [0,0-dimethyl 0-(2,4,5-trichlorophenyl) phosphorothioate] · Dimethoate or CL 12,880 (0,0-dimethyl S-methylcarbamoylmethyl phosphorodithioate) and Co-Ral or Bayer 21/199 [0,0-diethyl 0-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate]. The first two materials were given orally and the third applied dermally. In all cases, the peak secretion of insecticide in the milk occurred within 12 hr. after treatment. The highest milk residues were encountered with Trolene and the lowest

with Co-Ral. These experimental insecticides should not be tested on dairy cattle when the milk is to be marketed. This study was carried on in conjunction with metabolism and tissue residue studies on thees organo-phosphorothioates.

P28. Comparison of the Orange G dye and Kjeldahl methods for determining milk protein. J. M. TREECE, L. O. GILMORE, AND N. S. FECHHEIMER, Ohio Agricultural Experiment Station, Wooster, and The Ohio State University, Columbus.

A slight modification of the Orange G dye protein method of Udy was compared to the standard macro-Kjeldahl procedure, using 68-wk.-old composited samples of whole milk from Holstein, Jersey, and Guernsey cows of different ages and in different stages of lactation. From the unbound dye, which is measured colorimetrically, an estimate of the protein content can be made either graphically of by use of a predetermined constant. By using constants that disregard NPN, the sample means (3.16 and 3.16%) and standard deviations (0.62 and 0.60), respectively, for the dye and Kjeldahl methods, are not significantly different ($P < .001$). This shows that the dye method is as accurate as the Kjeldahl method and requires less than one-fourth the time. Furthermore, it is much less expensive than the Kjeldahl method, is specific for protein, and is easily adaptable to field use. This method is being further investigated, for use in estimating the casein fraction of milk.

P29. Milk preservative for solids-not-fat determinations.* M. L. NELSON, M. N. DEUTSCH, AND C. M. CLIFTON, University of Minnesota, St. Paul.

A survey was made of materials commonly used as preservatives for milk samples, in search of a suitable material for use in samples to be tested for solids-not-fat. Determinations were made by use of the small Watson lactometer, using 4-oz. samples, preheated and read after holding 5 min. in a thermostatically controlled water bath at 102° F. ±0.5°. Each preservative tested was evaluated in terms of fresh, unpreserved samples, by means of a split-sample technique. The qualities desired were a material which would not cause a significant change in specific gravity, and would preserve samples for at least seven days. Disodium phosphate, bichloride of mercury, sodium dichromate, and potassium dichromate produced from 0.26 to 0.90 increases in per cent S.N.F. over duplicate control samples. Zepherin failed to preserve samples seven days. Formalin used in the proportion of one part of formaldehyde to 1,500 parts of milk, produced slightly higher lactometer readings, averaging 0.03% S.N.F. ($P > 0.05$). This quantity of formaldehyde-preserved samples was taken under field conditions without refrigeration.

* This work was done in cooperation with the Dairy Husbandry Research Branch, USDA.

P30. In vitro cultivation of bovine mammary gland in tissue cultures. K. E. EBNER AND B. L. LARSON, University of Illinois, Urbana.

This paper is a preliminary report on investigations under way in this laboratory to study the biosynthesis of milk proteins in the secretary cells of the bovine mammary gland. As a part of this investigation, cell explants obtained by a collagenase dispersion of bovine mammary tissue were placed and maintained in culture using defined nutrients plus blood serum and/or plasma for the cultivation media. As far as the authors are aware, this is the first report of the in vitro proliferation of cells derived from the bovine mammary gland in tissue cultures. The various aspects of its cultivation will be discussed.

P31. Progress report on experimental stimulation of milk secretion.* C. W. TURNER AND H. L. RUPPERT, JR., University of Missouri, Columbia.

Study of the experimental induction of growth of the cow's udder and the initiation of milk secretion in normal and infertile heifers has been continued. Estrogens (100 μg. estradiol benzoate by injection or 10 mg. diethylstilbestrol orally) and 100 mg. progesterone have been administered daily for 180 days. Then, progesterone has been discontinued and 3 mg. estradiol benzoate injected for 14 days to initiate lactation. Some infertile heifers have conceived during the ensuing lactation period. Comparison of experimental maximum milk yield and normal milk production in these animals will be presented. A report on success of inducing experimental lactation after normal lactation will be presented. Why fremartin heifers generally fail to respond to experimental treatment will be discussed.

* These investigations were supported in part by grants of the U. S. Atomic Energy Commission [Contract No. AT(11-1)-301].

P32. Optimal hormone levels and lactation. C. E. GROSVENOR* AND C. W. TURNER, University of Missouri, Columbia.

A study was conducted to obtain uniform maximum milk yields per timed nursing in populations of lactating rats by the administration of optimal amounts of growth hormone (GH) and oxytocin, alone and in combination. Each rat on the 14th day of lactation was isolated from its litter for 10 hr., then replaced and allowed to nurse 30 min. Weight of milk thus obtained, expressed as per cent litter body weight, averaged 4.3% with normal distribution

for control animals. GH injected daily from the 7th to 14th days of lactation resulted in significantly greater yields (6.0%). Oxytocin injected at time of nursing into untreated rats similarly increased milk yields significantly (5.8%). Distribution curve of results following treatment with oxytocin or GH was shifted to the right and the range of values more constricted. There was further reduction in variance of milk yields in GH-treated animals injected at the time of nursing with oxytocin. Rather uniformly high milk yields were obtained. The results of this study indicate that individual variation among lactating animals may be greatly reduced by administration of optimal hormone levels, for it is probable that milk secretion and milk let-down is thereby enhanced in poor-lactating animals.

* Postdoctoral Fellow of N.I.H. This investigation was supported in part by a research grant from the Public Health Service.

P33. Protein-bound iodine and I^{131} extrapolation technique as methods of determining thyroid activity in dairy cows. F. B. Hamblin, J. E. Johnston, and G. Schrader, Louisiana State University, Baton Rouge.

Eight mature nonlactating cows (four Jerseys and four Holsteins) were placed in a climatic control chamber in a thermally neutral environment, in October, 1957. Serum protein-bound iodine was determined at five-day intervals, prior to I^{131} injection, by the Hycel method. Cows were injected subcutaneously with approximately 100 μc. of I^{131} per 100 lb. body weight. Maximum uptake of I^{131} by the thyroid occurred within 72 hr. Five days after initial injection, thiouracil was administered orally at the rate of 1 gm/cwt to partially prevent reutilization of I^{131}. Daily subcutaneous injections of l-thyroxine were begun after a release curve had been established. Thyroid counts were taken daily and corrected for decay and instrument adjustment. Dosages were increased (except for one cow which had to be decreased because of lower secretion rate) on alternate days until there was no further release of I^{131} from the thyroid. By this technique the thyroid secretion rate of individual cows was found to vary from 0.077–0.195 mg/cwt. The amount of PBI varied from 5.76–6.57 μg.%. Variability in thyroid secretion rate as measured by I^{131} was much greater than by PBI procedures. The two methods were found to have a positive correlation of 0.59.

P34. Turnover of I^{131}-labeled thyroxine in cattle.* B. N. Premachandra, G. W. Pipes, and C. W. Turner, University of Missouri, Columbia.

The determination of thyroxine secretion rate in cattle by replacement therapy involves repeated injections of thyroxine in increasing dosages until the release of I^{131} from the thyroid is completely inhibited. It was essential, therefore, to study the carry-over of unmetabolized hormone from one injection to another. I^{131}-labeled thyroxine was injected intravenously into eight dairy cows and calves, and daily radioactive measurements of the plasma and urine were made with a well counter. Thiouracil was administered to prevent reutilization of I^{131} by the thyroid of metabolized thyroxine I^{131}. Otherwise, reutilization would mask the true measurements of the biologic half-life of thyroxine in blood and consequently the appearance of I^{131} in the urine. The $T^1/_2$ of labelled thyroxine ranged from 2.0 to 2.75 days in plasma and the $T^1/_2$ for disappearance of I^{131} in urine ranged from six to eight days. From the rapidity of the disappearance of thyroxine from the blood, it is inferred that the estimations of thyroxine secretion rate in cattle are not exaggerated. This also indicates that thyroxine from thyroprotein is metabolized rapidly.

* These investigations were supported in part by grants of the U. S. Atomic Energy Commission [Contract No. AT(11-1)-301].

P35. Efficiency of absorption of thyroxine in thyroprotein from the gastrointestinal tract of lactating dairy cattle. J. P. Mixner and H. D. Lennon, Jr., New Jersey Agricultural Experiment Station, Sussex.

Seven lactating Holstein cows were used to determine the efficiency with which thyroxine in thyroprotein was absorbed from the gastrointestinal tract. The criterion of absorption efficiency was the elevation in the levels of plasma protein-bound iodine (PBI) by the orally administered thyroprotein, as compared to intravenously injected l-thyroxine. At different times each animal received intravenous l-thyroxine (25.2 mg.) and oral thyroprotein (25 g. containing 265 mg. of thyroxine), in order to have within-animal comparisons. At least two blood samples were collected from each animal during the disposal phase of peripheral thyroxine metabolism. The theoretical level of PBI at zero-time, with reference to injection of thyroxine and the oral administration of thyroprotein, was calculated. The PBI increase per milligram of thyroxine administered was then determined for the thyroxine from the two sources. With the injected thyroxine being rated as 100% efficient, the relative efficiency of absorption of the thyroxine in thyroprotein was evaluated. The mean efficiency of absorption of the thyroxine from the orally administered thyroprotein was 12.3±1.33%, with a range of from 8.30 to 17.4%, standard deviation being 3.53% and the coefficient of variation, 28.7%.

P36. Magnitude and variations in plasma PBI values in Ayrshire cattle at the West Virginia Agricultural Experiment Station. R. O. Asplund, G. A. McLaren, and I. D.

PORTERFIELD, West Virginia University, Morgantown.

Samples of blood ranging in number from three to eight were collected from two to five days apart, from each of 45 Ayrshires at 3-mo. intervals, from ages 3 to 18 mo. All samples were analyzed for plasma protein-bound iodine (PBI) by the method of Brown *et al.* PBI values were also determined on the dams of these animals. Unusually high PBI values were encountered in most individuals. Levels from 15 to 30 μg. per 100 ml. of plasma were common, and values as high as 75 μg. were obtained. These high levels were seasonal, occurring mostly in the spring, with a few in the late fall. This was found in all age groups, but none showed symptoms of hyperthyroidism. These results have been confirmed by other laboratories, using both the same and different analytical methods. The between-day variation of individuals was too large to detect significant differences between individuals. Some evidence indicates that grass silage may affect PBI levels in a positive manner.

P37. An isotope dilution technique for the determination of adrenocorticotropic hormone secretion rate in dairy cattle. W. F. WILLIAMS, University of Maryland, College Park.

Determination of the secretion rates of the various hormones is essential to the understanding of the endocrine physiology of dairy cattle and other animals. The development of methods for making these determinations is of importance to further advancements in this field. The usfulness of isotopic labeling of a protein pituitary hormone, ACTH, for the measurement of its secretion rate has been studied. An isotope dilution technique, involving a constant intravenous infusion of labeled ACTH and the determination of ACTH specific activity in periodic blood samples, has been developed for the secretion-rate determination. The possibility of the use of this technique for the determination of other hormonal secretion rates will be discussed.

P38. Some physiological responses of the bovine to dihydrotachysterol and parathyroid extract administration. K. A. KENDALL AND K. E. HARSHBARGER, University of Illinois, Urbana.

In a preliminary study with nonlactating Jersey and Guernsey cows, dihydrotachysterol was administered intramuscularly at levels of 6 and 9 mg. per animal while parathyroid extract at the rate of 1,000 units was administered. During a 24-hr. period, following administration, blood samples were taken, and fecal and urinary excretion rates were measured at regular intervals. Dihydrotachysterol appeared to have no diuretic effect, whereas urinary volume was doubled following parathyroid extract administration. Blood serum calcium and phosphorus showed little or no change when parathyroid was administered, whereas calcium appeared to increase slightly and phosphorus declined when dihydrotachysterol was injected. Both urinary phosphorus and calcium excretion rates were increased when parathyroid extract was injected; whereas, urinary phosphorus excretion in relation to calcium appeared to decline after the first 12 hr. following injections of dihydrotachysterol. Fecal calcium to phosphorus ratios in the dihydrotachysterol-treated cows appeared to increase from 1:3.5 to 1:6.3 at 12 hr. after injection. Fecal calcium and phosphorus values as related to parathyroid extract administration are being determined. It would seem from these preliminary observations that dehydrotachysterol and parathyroid extract, at the levels administered, did not produce similar physiological responses in the bovine.

P39. Release of ammonia nitrogen from uric acid, urea, and certain amino acids in the presence of rumen microorganisms. C. G. LOOPER AND O. T. STALLCUP, Arkansas Agricultural Experiment Station, Fayetteville.

The amino acids, arginine and glycine, and urea were each placed in artificial rumens in amounts so as to have equal nitrogen content. The ammonia in the flask containing glycine was not significantly different from a control flask containinly only inoculum. The release of ammonia from arginine was intermediate between that released from urea and the control. Morea (a urea-molasses-alcohol mixture) chicken litter (containing a high percentage of uric acid) and glycine were each fed to a fistulated steer, in conjunction with cottonseed hulls for roughage, in amounts so as to have equal nitrogen intake. The ammonia present in the rumen liquor, expressed as mgm/100 ml., at selected intervals after feeding for morea, chicken litter, and glycine, respectively, was as follows:

2 hours—	11.95,	13.40,	and 1.30
4 hours—	10.85,	8.1,	and 1.30
6 hours—	5.65,	3.40,	and 1.50
9 hours—	3.45,	1.30,	and 1.60
12 hours—	2.18,	1.00,	and 0.80

The ammonia released from glycine was approximately that released on a control diet when only cottonseed hulls were fed.

P40. The B-vitamin and N requirements of Bacterioides succinogenes, an important ruminal cellulolytic bacterium. HILDA CHU AND M. P. BRYANT, Dairy Cattle Research Branch, USDA, Beltsville, Maryland.

Previous studies on this organism showed that a fermentable carbohydrate, CO_2, and a two-component system of certain branched- and straight-chain volatile fatty acids were required for growth. The growth medium also contained minerals, purines, and pyrimidines, vitamin-free enzymatic hydrolysate of casein, and cys-

teine. Deletion of purines and pyrimidines had no effect on growth of five strains tested. Three strains gave optimum growth with only biotin of the B-vitamins present; the other two strains, with biotin and PABA. Biotin was a strict requirement of four strains and highly stimulatory to the other. PABA was highly stimulatory, but not a strict requirement of two strains. Casein hydrolysate could be replaced by a mixture of 19 amino acids or by cysteine-HCl alone. In the latter case, growth was good but sometimes delayed. NH_4^+ was required for growth, even when purines and pyrimidines and 19 amino acids were present in the medium. Glutathione replaced cysteine, but ascorbic acid and sodium sulfide were inhibitory. Work on N and S requirements are continuing. The results indicate that *Bacterioides succinogenes,* though a difficult organism to grow in pure culture, has a relatively highly developed ability to synthesize cellular constituents.

P41. Metabolism and absorption of butyrate-1-C^{14} by the perfused rumen. R. E. McCarthy, J. C. Shaw, Jeanne McCarthy, and S. Lakshmanan, University of Maryland, College Park.

Butyrate-1-C^{14} was added to the excised rumen of a 50-lb. goat (sacrificed 2 hr. after a normal feeding of hay and grain) at the beginning of an 80-min. perfusion study. Total production of rumen organic acids during perfusion in mg. was: acetic 641, propionic 306, butyric 268, valeric 107, formic 48, and lactic 306. Total increases (absorption) in perfusion blood organic acids substances in mg. were: acetic 363, propionic 117, butyric 107, valeric 25, and lactic 335; blood formic acid and glucose decreased 33 and 145 mg., respectively (ruminal tissue utilization), whereas no change occurred in blood acetone bodies. The final percentage distribution of radioactivity within the rumen showed some recycling of labeled butyrate. Of the total activity remaining, 62% was in butyrate, 25% in acetate, and 10% in propionate. Following perfusion, the activity in blood was 72% butyrate, 8.4% propionate, 7.9% acetate, and 9.7% lactate. There was little or no activity in blood formate, valerate, acetoacetate, β-hydroxybutyrate, carbon dioxide, and glucose (osazones). It appears that absorption of organic acids is a reflection of rumen production of these acids. Butyric acid is not converted to blood acetone bodies during absorption.

P42. Effect of KNO_2 and KNO_3 on in vitro cellulose digestion by bovine rumen microorganisms. V. H. Viebrock and C. P. Merilan, University of Missouri, Columbia.

Manometric studies were made on the in vitro utilization of a cellulose substrate (Solka-floc) by suspensions of rumen microorganisms from fistulated dairy heifers maintained on a roughage ration. The inclusion of low levels of KNO_3 in the reaction flasks stimulated gas production by 9.6, 5.8, and 3.3%, respectively, for concentrations of 0.125, 0.25, and 0.5% KNO_3 on a dry matter basis. However, the change in the portion of total gas production, which could be attributed to cellulose digestion, indicated 27.3, 32.4, and 27.8% increase in apparent cellulose digestion at these potassium nitrate concentrations. Higher levels of KNO_3, 1.0, 2.5, and 5.0%, resulted in decreased total gas production, as well as in 26.1, 48.9, and 80.0% decreases in apparent cellulose digestion. Potassium nitrite concentrations, ranging from 0.01 to 0.1% of dry matter, inhibited apparent cellulose digestion 7.9 to 67.0%, respectively, whereas 5.0% KNO_2 decreased the cellulolytic activity by 91%.

P43. Volatile fatty acid production and absorption in the perfused rumen. C. L. Davis, R. E. Brown, J. R. Staubus, and W. O. Nelson, University of Illinois, Urbana.

Three mature goats were starved 24 hr., then fed 2 lb. of hay and 1 lb. of concentrate. One hour after feeding the goats were anaesthetized and bled via carotid cannula until no further blood could be obtained. The rumens were removed and perfused first with saline rinse and then with the homologous heparinized blood. The average rate of VFA production was acetic, 2.61 g/hr; propionic, 1.16 g/hr; and butyric, 0.98 g/hr. The average absorption rates were acetic, 140 mg/hr; propionic, 72 mg/hr; butyric, 64 mg/hr. The low rates of absorption may be attributed to a slow rate of blood flow, which averaged 17 ml/min for the three perfusions. In contrast to reports in the literature, the relative proportions of VFA in rumen vein blood (A, 51, P, 26, B, 23) were nearly identical to those in the rumen fluid (A, 55; P, 22; B, 23). Glucose utilization by rumen tissue was demonstrated by A-V glucose differences of 6–10 mg.%. When venous blood was recirculated during one perfusion, there was a threefold increase in rate of blood flow, but there was no VFA absorption into the recirculated blood and no glucose uptake by the rumen tissue.

P44. Effects of various feeds on volatile fatty acid production in vitro. R. G. Hinders and G. M. Ward, Colorado State University, Fort Collins.

Rumen bacteria obtained from cows on rations of (I) brome grass hay, (II) brome grass hay and grain, and (III) alfalfa hay and grain were used for in vitro studies of volatile fatty acid (VFA) production. VFA content and pH were determined using excellent alfalfa, poor alfalfa, brome grass hay, mixed alfalfa and grass hay, mixed excellent alfalfa and concentrate, and concentrate alone as the substrates. Rumen bacteria from animals on Ration III with excellent alfalfa as the substrate

produced the highest concentration of acids with 140.2 μM/ml (50.3% acetic, 34.7% propionic, and 15% butyric and higher acids). The lowest value was produced by Ration I bacteria with poor alfalfa as the substrate (107 μM/ml; 52.7% acetic, 29.8% propionic, and 17.4% butyric). The acetic, propionic, and butyric acid percentage of VFA and the pH, respectively, for Ration I was 58, 24, 16, and 6.6; for Ration II—52.7, 29.9, 17.4, and 6.4, and for Ration III, 63.8, 20.2, 16.0, and 6.3.

P45. Effect of ethanol on rumen fermentation. T. R. Lewis, R. S. Emery, and J. P. Everett, Michigan State University, East Lansing.

Ethanol (800 ml., 47.5%) was administered via rumen fistula during the once-daily feeding of, (1) 14 lb. timothy hay plus 4 lb. of equal parts ground corn and corn cobs or, (2) ration 1 with 3% urea replacing corn or, (3) 20 lb. alfalfa hay, to twin 1,100-lb. cows. Each ration was fed with and without alcohol to each cow for 14-day periods. Four per cent of ground ration was added to coarsely strained rumen liquid and incubated for 3 hr. in vitro. Ethanol was not fermented in vitro but disappeared from the rumen with a half-time of 2 hr. A maximum rumen ethanol concentration of 500 mg. per cent was indicated. Blood contained 75 mg. per cent ethanol 2 to 4 hr. after administration. Ethanol depressed rumen dry matter content, and utilization of radioactive inorganic sulfate for cystine synthesis in vitro without affecting in vitro ammonia utilization or synthesis of methionine or glutathione. Total gas production in vitro was depressed by ethanol.

P46. Efficiency of rumen fermentation as affected by corn and adaptation to ration. R. S. Emery, C. K. Smith, T. R. Lewis, and June DeHate, Michigan State University, East Lansing.

A 900-lb. rumen fistulated steer was fed daily *(1)* 18 lb. alfalfa hay, or *(2)* Ration 1, with corn substituting for 10% of the alfalfa T.D.N., or *(3)* corn substituting for 20% of the alfalfa T.D.N. Inocula (200 ml. coarsely strained rumen liquid), taken from two to five and from 19 to 21 days after each ration change, was incubated in a flask with 10 g. finely ground Ration (1) or 9.4 g. Ration (2) or (3). Three milliliters sulfuric acid were added to separate flasks after 0, 3, and 6 hr., to stop the fermentation. Inoculum from Ration (1) was also fermented with each substrate. Fermentation products were converted to their caloric equivalents, assuming 0.0035 Cal. per milliliter CO_2 produced. Fatty acid production was increased by corn and by adaptation to the ration, largely owing to increased acetic acid production. Caloric rations of CO_2 and CH_4 were 2.3 and 1.6 for the first and second 3 hr. of fermentation for the eight trials. The efficiency ratios (calories of volatile fatty acids to calories of gas) were 18.7, 17.4, and 22.5 for Rations (1), (2), and (3). These were not affected by adaptation to the ration.

P47. Physical and chemical changes in bovine saliva and rumen liquid with different hay: grain ratios. C. K. Smith, R. S. Emery, R. M. Grimes, C. F. Huffman, and C. W. Duncan, Michigan State University, East Lansing.

Saliva was collected at the cardial orifice and rumen liquid samples were taken before feeding and 3 hr. after feeding from two cows on 20, 40, 60, 80, and 100% hay rations. The viscosity (mean 104 cp.) was greater with 20 and 80% hay rations than with 60 and 100% hay rations. The viscosity after feeding was about twice that obtained before feeding. The pH (mean 8.21) tended to increase the proportion of hay increased. Ash (mean 0.7337 g/100 ml.) tended to be higher at both extremes of the hay:grain ratio. Organic matter (mean 0.3854 g/100 ml.) tended to be low at both extremes of the hay: grain ratio. Total nitrogen was 20.05 mg.% for the 20% hay rations and averaged 14.88 mg.% for the 40–100% hay rations. Hexosamine (mean 4.25 mg.%) was higher on the high grain ration. The rumen liquid samples were centrifuged at 25,000 G and the supernatant analyzed. The dry matter mean was 1.71% and the ash content mean was 0.79%. Sodium (mean 234 mg.%) increased with increasing hay content. Hexosamine (mean 36 mg.%) tends to increase with increasing grain concentration. Eighty per cent ethanol soluble dry matter (mean 1.32 mg.%) was greater after feeding. The total nitrogen (mean 37.1 mg.%) was higher on the 20% hay rations and higher after feeding.

P48. Relationships among acetate, propionate, and butyrate metabolism in ruminant liver slices. G. I. Pritchard and S. B. Tove, North Carolina State College, Raleigh.

Acetate, propionate, and butyrate metabolism were studied by incubating C^{14}-labeled substrates with buffered liver slices from a ruminant starved for from six to eight days. Presence of propionate reduced the incorporation of C^{14} from carboxyl-labeled acetate into carbon dioxide, lipids, and protein. Acetate, glucose, or butyrate increased the incorporation of C^{14} from carboxyl-labeled propionate into carbon dioxide, glucose, succinate, malate, and fumarate; whereas, citrate caused no differences. The effect produced by butyrate was greater than that produced either by acetate or by glucose. Apparently, a relationship exists between propionate and butyrate metabolism. This relationship can not be explained on the basis of energy, citric-acid-cycle activity, or competition for the activating enzyme.

P49. Fertility of bull semen stored for one and two days at 5° C. in 20% yolk-citrate-glycine-glucose extenders. R. H. FOOTE AND D. C. YOUNG, Cornell University, AND H. O. DUNN, New York Artificial Breeders' Cooperative, Inc., Ithaca, New York.

In experiments at Cornell, two extenders (CU-16 and CUE) were found to be superior to a 50% yolk-citrate control (C) in maintaining motility of bull spermatozoa. CU-16 consisted of one part of yolk to four parts of buffer containing 1.45% sodium citrate, 1.25% glucose, and 0.937% glycine. CUE consisted of one part of yolk to four parts of a pH-adjusted buffer containing 1.45% sodium citrate, 0.21% sodium bicarbonate, 0.04% potassium chloride, 0.30% glucose, and 0.937% glycine. All extenders contain sulfanilamide, penicillin and dihydrostreptomycin. To test fertility, semen from four Holstein bulls was extended at the rate of ten million motile sperm per milliliter of each extender. Motility studies indicated that there were between eight and nine million motile sperm per milliliter at the time of insemination. Semen was packaged in polystrene tubes. For one-day-old semen the number of first services and the 60- to 90-day nonreturn rate for C was 603 and 78%; for CU-16, 650 and 77%, and for CUE, 601 and 79%. For two-day-old semen the corresponding figures were 373 and 71, 399 and 73, and 386 and 75%.

P50. Prolonged survival of bovine sperm in the Illini variable temperature diluent. N. L. VANDEMARK AND F. D. BARTLETT, JR., University of Illinois, Urbana.

In semen ampuled with the original IVT diluent (*J. Dairy Sci.*, 40; 438. 1957), sperm motility in split ejaculates from four bulls decreased from 71 to 63% in seven days to 14% after 20 days at 40° F. With higher levels of glucose, egg yolk, and the addition of catalase to the diluent, sperm motility dropped from 71 to 67 and 43% in seven and 20 days, respectively. Further improvement in sperm survival resulted with higher levels of sodium bicarbonate. In storage at 40° F., sperm motility decreased only from 71 to 63% in 20 days. Even at 40 days and 60 days, 57 and 40% actively motile sperm were found, respectively. Storage at 40° F. gave better survival than at 80° F. Fertility of semen stored at room temperature in the original IVT diluent has not been satisfactory in large-scale usage (*Proc. NAAB*, p. 112, 1957). Insemination of 326 cows with semen stored from one through seven days at 40° F. in the IVT diluent, with increased glucose, egg yolk, and added catalase, has resulted in 10–20% higher 60-day nonreturn rates throughout the week over those in the earlier large-scale trials. Fertility tests with semen stored in the diluent which has given even longer sperm survivals are in progress.

P51. A comparison of two diluents in single-service glass ampules for routine artificial insemination of cattle. A. F. MCFEE, L. J. BOYD, AND E. W. SWANSON, University of Tennessee, Knoxville.

The possibility of improving conception rate of fresh liquid semen through four days of age, by providing semen in single-service sealed glass ampules instead of in the common multiple-service plastic vials was investigated. Split ejaculates were diluted in the Illini (IVT) and yolk-citrate (YC) diluents and sealed in glass ampules before cooling. Control YC was also packaged in 5-cc. plastic vials. Cooled semen was shipped from the laboratory to three groups of technicians each day, alternating the two diluents between two of them. Semen was collected from one bull of each of three breeds at four-day intervals for 40 days. Motility at six days averaged 54% in IVT and 57% in YC in ampules. From 160 to 200 services were made each day to each semen treatment. Nonreturn rates (38–78 day) for IVT, YC ampule, and YC vial were, respectively, 75.8, 76.0, and 79.7% at one day; 81.6, 64.0, and 71.1% at two days; 77.7, 51.7, and 64.1% at three days, 62.3, 49.6, and unchecked, at four days. These results indicated *(1)* that there was no advantage to packaging the YC-diluted semen in sealed samples and *(2)* that the ampuled IVT was more efficient than the ampuled YC and YC in vials after the first day's use.

P52. Fertility of bull semen stored at room temperature for six days. H. O. DUNN, New York Artificial Breeders' Cooperative, Inc., AND R. H. FOOTE, Cornell University, Ithaca, N. Y.

In July, 1957, semen from six bulls was extended at a rate of ten million motile sperm per ml. in 50% yolk-citrate at 5° C. and in carbonated Illini Variable Temperature extender at ambient temperature. Approximately 200 technicians made the inseminations with one-day-old control semen shipped in plastic vials and with one- to six-day-old carbonated semen shipped in sealed ampules at ambient temperature.

The 60- to 90-day nonreturn rate for one-day-old semen was 76% for 963 first services. The number of services and nonreturn rates for one- to six-day-old carbonated semen were 152 and 69, 161 and 45, 139 and 33, 121 and 26, 126 and 25, and 139 and 21%, respectively. The total services and average nonreturn rate for the carbonated semen were 838 and 38%. The average nonreturn rates by bull for the control semen were 73, 78, 74, 79, 78, and 77. For the carbonated semen, the corresponding rates were 27, 28, 39, 52, 39, and 45. High ambient temperatures, defects in ampules, and the 2-hr. processing procedure were considered as contributing factors to the low fertility results with the carbonated semen.

P53. Livability of bovine spermatozoa at room temperatures in a coconut milk-citrate-calcium carbonate diluent. CHARLES NORMAN, C. E. JOHNSON, AND I. D. PORTERFIELD, West Virginia University, Morgantown.

A series of metabolic studies performed on three pooled samples of bovine spermatozoa, maintained in a coconut milk-citrate-calcium carbonate medium (1:1 mixture of boiled coconut milk and a 4.32% sodium citrate solution, containing 100 mg. % calcium carbonate, 80 mg. % penicillin, and 90 mg. % dihydrostreptomycin sulfate), indicated that an appreciable amount of metabolic activity could be restored by resuspending the spermatozoa in fresh diluent at the end of six days of storage at room temperatures (23–27° C.). Reactivation of metabolic activity without renewing the diluent was also accomplished by adding $CaCO_3$. Measurements of sperm respiration, anaerobic glycolysis, motility, % live sperm, and pH were made at 3, 76 and 150 hr. after semen collection. Renewal of diluent after 76 hr. of storage showed recovery values of 71, 74, and 83% with respect to sperm O_2 uptake, anaerobic glycolysis, and number of unstained sperm. After six days of storage, renewal of diluent produced recovery of 60, 65, and 84% of the initial values for O_2 uptake, anaerobic glycolysis, and number of live sperm, respectively. Vigorous translatory motility was observed in all reactivated samples.

P54. Comparison of skimmilk-egg yolk-glycerol, egg yolk-citrate-glycerol, and homogenized whole milk-glycerol as extenders for bovine semen. B. W. PICKETT, M. C. JOHNSTONE, W. A. COWAN, AND PAUL HELLER, University of Connecticut, Storrs.

A laboratory study was initiated to compare the storage life of bovine spermatozoa diluted in heated skimmilk-egg yolk-glycerol (A), egg yolk-citrate-glycerol (B), and heated, homogenized whole milk-glycerol (C). The procedure of Sikes and Merilan (1958) was followed for preparation of (A). Concentrations of the skimmilk-egg yolk-glycerol were 82, 10, and 8%, respectively. Split semen samples were diluted in (A), (B), and (C), duplicate samples were stored at 5° C. and at from 0 to −3° C. Results from a limited number of samples observed daily indicated that extenders (A), (B), and (C) maintained a motility rating of one or above (0–4 scale) for 8.5, 5, and 6 days at 5° C., and for 12.5, 5, and 7.5 days at 0 to −3° C., respectively. Samples stored in 2-ml. ampules were superior to those stored in tubes routinely used for shipping liquid semen. Dead-live strains were inconsistent and did not show the differences among extenders that would be expected from the motility ratings. Field trials are in progress to compare the breeding efficiency of Extenders (A) and (B).

P55. Sperm reserves of dairy bulls as determined by depletion trials and postslaughter sperm counts. J. O. ALMQUIST, R. P. AMANN, AND W. T. O'DELL, The Pennsylvania State University, University Park.

Sperm numbers in billions for the combined right and left head, body, and tail of epididymis, ductus deferens, and ampulla of 8 bulls (from 2 to ten years old), sexually rested for 30 days before slaughter, were: 20.3, 4.8, 37.3, 2.4, and 6.1, respectively, totalling 70.9±14.3. In ten bulls depleted just prior to slaughter, the extragonadal reserves, including ejaculated sperm, averaged 69.9±23.0 billion. An average of 37.2 billion sperm was removed immediately before slaughter, representing 53.4±3.6% of the sperm reserves. Thus, depletion trials constitute a reliable estimate of sperm reserves. The 37.2 billion sperm removed agrees closely with the value of 38.7 billion sperm obtained by depleting 27 additional bulls. Depleted bulls contained 23% fewer sperm in the head of the epididymis than rested bulls. There was no increase in the proportion of sperm showing protoplasmic droplets ($< 2\%$) with successive ejaculations. Nonsignificant correlations were found between extragonadal reserves and paired testis weight (±0.247) and paired epididymis weight (+0.329). Testis sperm counts of ten bulls averaged 18.4±4.0 billion per testis, or 56.3±13.6 million per gram of testis.

P56. Sperm production and fertility of dairy bulls at high-collection frequencies with varying degrees of sexual preparation. J. O. ALMQUIST, E. B. HALE, AND R. P. AMANN, The Pennsylvania State University, University Park.

Minimum values for sperm production of three Holstein bulls two, five, and eight years old (based on sperm output when collected 60 to 70 times per week for from 5 to 6 wk. following depletion of sperm reserves) were 7.7, 6.8, and 7.2 billion per day, respectively. Sperm output for three mature bulls collected six times weekly with intensive sexual preparation (including three false mounts for each ejaculate) was 5.1, 6.5, and 7.7 billion per day. These latter values represent an increase of about 30% over output with less intensive preparation (with or without one false mount). Thus, daily sperm output values on six ejaculates per week, collected with adequate sexual preparation, approached the minimum sperm production obtained at very high collection frequencies. With one bull this value was as high as 90% of the estimated sperm production (7.2 billion per day). Observations with bulls collected twice weekly also indicate greater sperm output following three false mounts rather than one. The fertility of three bulls collected six times per week for 8 to 14 mo. was 70, 74, and 77% 60- to 90-day nonreturns to 281, 286, and 656 first-service inseminations, respectively, starting 6 mo. after initiating the six-ejaculates-per-week regime.

P57. Effects of daily ejaculation on sperm output, fertility, and libido of dairy bulls. H. D. Hafs, R. S. Hoyt, and R. W. Bratton, Cornell University, Ithaca, New York.

Semen was collected once a week from six dairy bulls for a 4-wk. preliminary period, after which three bulls were randomly chosen for collecting once a day. During the first 15 wk. of the experimental period, the averages for ejaculate volume, per cent motile, sperm concentration, motile sperm per ejaculate, and total sperm per ejaculate for the bulls collected once a week were 8.37 ml., 64%, 1.68×10^{9}, 8.93×10^{9}, and 13.84×10^{9}, respectively, and for the bulls collected once a day were 5.43 ml., 67%, 0.81×10^{9}, 3.20×10^{9}, and 4.60×10^{9}, respectively. The motile sperm output per week for the daily bulls averaged 22.4×10^{9}. During this period, daily collection did not measurably affect libido or body weight. For the first 10 wk., the average 28–35 day nonreturn rate was 81.2% for 3,654 first services to the bulls collected once a week and 82.7% for 1,317 first services to the bulls collected once a day. Laboratory studies of the pH, staining properties, and abnormal sperm of the raw semen, and the livability of the sperm during storage at −79° C., 5° C., and room temperature, showed no consistent differences between the two collection frequencies.

P58. Microbiological and chemical changes in grass silage. C. W. Langston, Herbert Irvin, C. H. Gordon, Cecelia Bouma, H. G. Wiseman, C. G. Melin, and L. A. Moore, Dairy Cattle Research Branch, and J. R. McCalmont, Agricultural Engineering Research Branch, USDA, Beltsville, Maryland.

Three cuttings of orchard grass and alfalfa forages ensiled in 4- by 8-ft. experimental silos were treated, to obtain a wide range of quality. Samples were taken from the silos at different stages of fermentation for bacteriological and chemical analysis. The silages were divided into good and poor quality on the basis of pH, ammonia nitrogen, lactic acid, butyric acid, and spore counts. The lactic acid bacteria reached about the same maxima in both the good- and poor-quality forages, and the few silages that showed relatively high initial numbers of lactic acid bacteria were apparently no better in quality than those with few or no lactic acid bacteria. The primary difference in quality in respect to the occurrence of microorganisms was associated directly with the increase in spore-forming anaerobes. All the poor-quality silages showed substantial increases in these organisms. Microorganisms in the good-quality silages produced large quantities of lactic acid, along with fair amounts of acetic and succinic acids. Organisms in the poor-quality silages produced butyric acid at the expense of lactic acid. Acetic, succinic, and propionic acids occurred along with butyric acid.

P59. Preservatives for alfalfa silage. R. J. Baker and H. H. Voelker, South Dakota Agricultural Experiment Station, Brookings.

Third-cutting alfalfa, 75 and 60% moisture, was preserved in glass jars, 60-lb. capacity plastic bags, and 1.5-ton plastic silos. Preservative rates included molasses 4%, lactobacillus plus molasses (?%?), cane sugar 2.4%, sodium chloride 2.5%, ground barley 10%, sprouted barley 10%, ground corn 10%, and no preservative. Total aerobe, proteolytic, lactobacillus, coliform, and pH determinations were made as preservation progressed. The pH dropped fastest and remained lowest (4.3–4.7) in lactobacillus plus molasses, molasses alone, sugar, and sprouted grain silages. Sprouting accentuated pH declines (sprouting should activate amylase, break down starch for quicker lactobacilli use). Salt and control silages were high in pH. Most offensive silage was high-moisture, no-preservative alfalfa in poorly sealed "silos." Forty-two dairy calves were allotted to treatment groups and individually fed ad libitum some of the high-moisture silages from 30 to 148 days of age, testing the feeding values of the preservatives. The rates of consumption and average daily gains (lb.) to date, respectively: sprouted barley first, 1.79; sugar second, 1.91; ground barley third, 1.62; molasses fourth, 1.69; no preservative fifth, 1.66, and salt sixth, 1.52.

P60. Effects of wilting and preservatives on composition of legume-grass silage in miniature silos. R. S. Allen and C. F. Foreman, Iowa State College, Ames.

Nonwilted (69% moisture) and wilted (58% moisture) second-crop alfalfa from the same field were ensiled with no preservative, with added sodium metabisulfite (8 lb/ton), and with added ground corn (100 lb/ton) in concrete miniature silos (2 ft. in diameter, 6 ft. high). Each treatment was replicated. Compaction was accomplished by tramping during filling and thereafter by a constant pressure of 80 lb/sq foot. Samples were taken for chemical analyses at the time of filling and when the silos were emptied from 3 to 3½ mo. later. Wilting resulted in increases (dry matter basis) in amino nitrogen, nonprotein nitrogen, and reducing sugars in the forage as ensiled. Silages resulting from wilted material were substantially lower in amino nitrogen, ammonia nitrogen, and nonprotein nitrogen than the silages from nonwilted forage. Reducing sugar levels were considerably higher, whereas pH values tended to be somewhat lower in wilted silages. Within the group of nonwilted silages, the sodium metabisulfite-treated material contained less ammonia nitrogen and much more (six- to tenfold) reducing sugar than the nonpreserved or corn-preserved silage. Amide nitrogen value were essentially the same in all silages.

P61. Toxic gases from silage. L. C. WANG, R. H. BURRIS, AND R. P. NIEDERMEIER, University of Wisconsin, Madison.

Because newly filled silos may release gases hazardous to livestock and to man, it is important to determine the types of gases produced and the time-course of their production in full-scale silos. Representative samples of silage gases, free of air contamination, were obtained through a fine-bore polyethylene tube supported in a vertical pipe inserted to a height of 5 ft. in a 12- by 40-ft. tower silo filled with corn silage. Periodic analyses of gas samples with a mass spectrometer indicated a rapid rise in the concentrations of CO_2 and NO, a slow rise in N_2O, and a rapid decrease in O_2, N_2, and argon, with time. Mass analysis and colorimetric analysis indicated little production of NO_2 in the silo. This toxic gas is formed from NO when it comes in contact with the oxygen of air. The NO rose to a concentration of ten volume % in the silo gases within 24 hr. and declined to less than 1% within 42 hr. The N_2O continued to rise as the NO disappeared. N_2 disappeared less rapidly than argon, for N_2 was formed by denitrification in the silo. Denitrification can account for considerable losses of nitrogen from silage.

P62. Nutrient losses and feeding values of wilted and direct-cut forages stored in bunker and tower silos. C. H. GORDON, C. G. MELIN, W. C. JACOBSON, H. G. WISEMAN, E. A. KANE, AND J. C. DERBYSHIRE, Dairy Cattle Research Branch, AND J. R. MCCALMONT AND D. T. BLACK, Agricultural Engineering Research Branch, USDA, Beltsville, Maryland.

First-cutting forage, principally orchard grass, was harvested simultaneously from the same fields and stored as *(1)* wilted forage in a tower silo, *(2)* direct-cut forage in a bunker silo, and *(3)* as a direct-cut forage in a tower silo. Both direct-cut forages were treated with 8 lb. sodium metabisulfite per ton. Silage temperatures were recorded for the tower-stored silages. Composition changes, nutrient losses, digestibility, and feeding value for dairy cows were determined for each silage. Top spoilage losses were minimized in all silos by the use of weighted plastic covers. Total dry matter losses were 13.7% in the wilted tower-stored silage, 14.8% in the direct-cut, bunker-stored silage, and 20.9% in the direct-cut, tower-stored silage. Higher seepage losses accounted for a large part of the greater total losses in the latter silage. All silages were of good chemical quality, with pH values of 4.2–4.4. The wilted silage had a higher content of the organic acids measured. Wilted silage dry matter was consumed at the rate of 23.3 lb. per cow daily which, with grain supplement, produced 1.8 lb. of live weight gain and 24.5 lb. of FCM daily. Respective values for bunker-stored silage were 20.1, 0.7, and 25.1, and for direct-cut, tower-stored silage, 20.5, 1.3, and 23.5.

P63. Feeding value for milking cows of oat silages cut at three stage of maturity. F. A. MARTZ, C. H. NOLLER, D. L. HILL, AND M. W. CARTER, Purdue University, Lafayette, Indiana.

The feeding value of oat silages cut at three stages of maturity—*(1)* boot, *(2)* milk, *(3)* dough—and *(4)* alfalfa-ladino-brome grass silage were compared. The silages were direct-cut with 6–8 lb. sodium metabisulfite per ton and stored in upright silos. Twelve lactating dairy cows were used on three 4×4 Latin-squares, with an extra period to measure carry-over effect. A seven-day preliminary period was followed by an eight-day experimental period. The silages were fed ad libitum plus 0.3 lb. hay daily per 100 lb. body weight and an 18.9% crude protein grain at 1:6. The silages contained 79.0, 76.5, 70.1, and 74.5% moisture and had a pH of 4.40, 4.65, 4.60, and 4.39, respectively. The titratable acidity was 0.79, 0.56, 0.84, and 1.11%. The treatment means for dairy FCM were 23.3, 21.1, 22.2, and 21.7 lb., respectively. The silage treatments were statistically significant ($P \leq 0.05$). The average daily silage dry matter intakes were 21.9, 21.0, 25.4, and 19.9 lb., respectively. The dry matter intakes were significantly different ($P \leq 0.01$). The average body weight changes per cow per 15-day period were −2.5, 0.0, +10.5, and −4.5 lb., respectively. There were no apparent carry-over effects.

P64. Soilage or silage for lactating dairy cows? C. F. FOREMAN, R. S. ALLEN, AND A. R. PORTER, Iowa State College, Ames.

A comparison was made between first-crop alfalfa silage (bunker silo, 10 lb. sodium metabisulfite/ton) and second-crop alfalfa (chopped and fed fresh daily), when fed to lactating cows. Grain was fed at a constant level, based on individual milk production during the 2-wk. pre-experimental period on pasture. Average pounds daily milk production, per cent total solids (TS), and per cent butterfat (BF) during the pre-experimental period were 45.7, 11.29, 3.10, and 45.6, 10.87, 2.87, for cows on soilage and silage, respectively. Average pounds daily milk production, % TS, and % BF during the 8-wk. experimental period for soilage- and silage-fed cows were 42.3, 11.50, 3.03, and 36.5, 11.10, 3.04, respectively. Most of the decrease in milk production on silage occurred during the 1st wk. of the experiment. The mean per cent protein, crude fiber, and N.F.E. on a dry basis was 20.55, 26.04, and 42.41 for soilage, and 17.44, 31.85, and 35.85 for silage. A percentage increase in crude fiber, and a decrease in protein and N.F.E. in the silage, occurred as the experiment progressed. Variations in milk production and % TS, and in the composition of soilage and silage, will be discussed.

P65. Milk production correlated with quality and quantity of daily herbage intake. A. L. BRUNDAGE AND W. J. SWEETMAN, Alaska Agricultural Experiment Station, Palmer.

Six lactating dairy cows were used during the grazing season of 1955, to study the correlation of herbage intake and milk production under rotational and strip grazing. Fecal samples were obtained at 7:30 A.M. and 4 P.M. daily and composited into consecutive three-day samples in the manner of a running average. The combined Cr_2O_3-chromogen procedure was employed, to compute apparent dry matter digestibility and total intake. Multiple correlation analyses were computed for each cow, using time, digestibility, fecal output, digestible intake, bi-daily variation in liveweight, and milk production as variables. Under strip grazing, nearly all the variation in daily milk production was attributed to time. Time accounted for less of the variation in milk production under rotational grazing. A highly significant portion of the remaining variation was attributed to fluctuation in digestibility and digestible intake. These data suggest that digestibility and digestible intake are limiting factors under rotational grazing but not under strip grazing.

P66. Forage and milk yields from alfalfa under three different harvesting systems. R. M. PORTER AND S. R. SKAGGS, New Mexico Agricultural Experiment Station, State College.

The experiment was set up to determine the relative yield of forage, labor costs, and milk yield when alfalfa was used for pasture, soiling crop, or soilage. A 10% increase in yield was obtained by mechanical harvesting. No bloat problem was experienced using the California system of controlled grazing, and excellent consumption of available forage was obtained. There was no significant difference in milk yield while utilizing alfalfa as pasture, silage, or soiling crop. Pasturing of alfalfa was the most economical, but it is felt that this method would have limited use.

P67. Fertility of frozen semen stored in plastic and glass ampules and of first and second ejaculates. H. H. BRUGMAN AND R. P. SCHMIDT, University of Maine, Orono.

The Connecticut and Massachusetts Artificial Breeding Cooperatives supplied the semen. Each ejaculate was split and half was placed in glass ampules and the other half in plastic 1-cc. polybulbs. Connecticut discontinued the use of polybulbs because they floated. Connecticut had 235 first services. Sixty–ninety day nonreturn for the first ejaculate was 48.0% and for the second was 57.4%. Massachusetts had 492 first services. Sixty–ninety day nonreturn for the first ejaculate (glass and plastic) was 50.2%; for the second ejaculate (glass and plastic), 65.4%, both ejaculates in plastic, 59.9%, and in glass, 56.0%. The difference in conception rate between first and second ejaculates was highly significant ($P < 0.01$). Difference between plastic and glass ampules was not significant ($P > 0.05$). This study indicates that plastic polybulbs or glass ampules can be used satisfactorily for storing frozen semen in CO_2 and alcohol. Polybulbs are difficult to store because they float. Conception data showed a highly significant difference in favor of the second ejaculate.

P68. Addition of progesterone and estrogen to bovine semen. R. E. STAPLES, W. HANSEL, R. H. FOOTE, AND H. O. DUNN, Cornell University, Ithaca, New York.

Progesterone (1–10,000 μg. per ml.) and estradiol (0.01–800 μg. per ml.), alone and in combination, were added to bovine semen extended with yolk: citrate to ten million motile sperm per milliliter. No differences attributable to hormone treatments were observed in motility or longevity of the semen during storage at 5° C. Two fertility trials were conducted which involved a total of 3,245 dairy cows and eight dairy bulls. In the first trial, 20 μg. of progesterone and 1 μg. of estrogen per milliliter of extended semen were used, both singly and in combination; in the second, 5,000 μg. and 200 μg. per ml. were used, respectively. The 60–90 day per cent nonreturns in the first trial were as follows: Control—75, progesterone—73, estradiol—74, and progesterone plus estradiol—73. In the second trial, the 60–90 per cent nonreturns were: Control—70, progesterone—71, estradiol—72, and progesterone plus estradiol—69.

P69. Density of "sampler" bulls. R. B. BECKER AND P. T. DIX ARNOLD, Florida Agricultural Experiment Station, Gainesville.

Records of 1,002 dairy bulls started in artificial use between 1942 and 1951 as yearlings and 2-yr.-olds, were analyzed, to determine the average fate of so-called sampler bulls. Of the 317 bulls culled, low production of daughters accounted for 40%; poor conformation, 22%; replaced by better bull, 18%; unpopular, 10%, and the remaining 10% for bad disposition, inbreeding, expiration of lease, stud disbanded, or never used. The average age at culling was 5.5 yr. old. One bull was 1.1 yr. old, and ten bulls were past 10 yr. old before this final decision was made. Low breeding efficiency terminated use of 48% of 460 bulls removed from artificial use by natural causes and some before they had daughters in milk. Of these 1,002 bulls, 32% proved unsatisfactory; 46% left service for natural causes at various ages, and the remaining 22% were still in use when from 6 to 13 yr. of age.

P70. Results from breeding cows with frozen semen thawed for various lengths of time. VICTOR HURST AND J. W. KELLY, South Caro-

lina Agricultural Experiment Station, Clemson.

During the spring and summer of 1957, heifers and cows in the Clemson dairy herd were inseminated with frozen semen after it had been thawed for different periods of time. Results of breedings based on pregnancy examinations at 3 mo. were as follows: Semen used within 0.5 hr. after thawing, 54 inseminations, 43% pregnant; semen used 1 hr. after thawing, 48 inseminations, 38% pregnant; semen used 2 hr. after thawing, 13 inseminations, 31% pregnant; semen used 3 hr. after thawing, five inseminations, 20% pregnant; semen used 5 hr. after thawing, one insemination, 100% pregnant. By eliminating low-conception bulls (below 33% on pregnancy diagnosis) the conception rates in the 0.5-, 1-, and 2-hr. groups were raised to 51, 52, and 60%, respectively.

P71. Some aspects of the role of glycerol in the metabolism of bovine semen. R. J. FLIPSE AND A. A. BENSON, Pennsylvania Agricultural Experiment Station, University Park.

Bovine spermatozoa were washed free of seminal plasma, then incubated aerobically with glycerol-1-C^{14} in Ringer-phosphate. CO_2 was collected during incubation; after the 3-hr. incubation period the cells were washed free of the medium and extracted, first with 0.6 N perchloric acid (PCA), then with 95% ethanol, and finally with ethanol-ether. Of the recovered radioactivity, 65% was in CO_2, 8% in the PCA extract, 22% in the combined ethanol and ethanol-ether extracts, and 5% in the residue after extraction. The PCA-soluble fraction was paper-chromatographed and most of the activity was attributed to lactate, although radioactivity also was noted in succinate and in several unidentified compounds. Components of the phospholipids of bovine spermatozoa were characterized by activation analysis. Phospholipids were extracted from plasma-free spermatozoa, hydrolyzed with methanolic KOH, and the hydrolysate chromatographed on paper. After two-dimensional development, the chromatogram was irradiated at a flux of 5×10^{11} neutrons/cm^2/sec for 5 hr. The radioactivated phosphorus was then used to locate glycerophosphate esters. By this procedure, glycerophosphorylcholine, glycerophosphorylethanolamine, glycerophosphorylserine, and diglycerophosphate were identified as derivatives of sperm phospholipids. The function of these esters in the metabolism of spermatozoa will be discussed.

P72. Comparison of filtered versus nonfiltered bovine semen after extension and low-temperature storage. M. A. BROWN AND J. C. JOHNSON, A & M College of Texas, College Station.

An apparatus utilizing pima-cotton broadcloth as a filter allowed, on the average, 32% of spermatozoa to pass through the filter. Nonfiltered control portions of split ejaculates contained an average of 59% live sperm, whereas filtered aliquots of the samples contained an average of 77% live cells. The filter was effective in removing a portion of the dead and abnormal spermatozoa from bovine semen samples. Extended semen, from which an appreciable portion of dead and abnormal cells had been removed, had an average survival rate of 20% after glycerolization, equilibration, and storage at −79° C. for seven days. Nonfiltered semen similarly treated had an average survival of 39%. It appears that one or more semen components that contribute to improved sperm livability during low-temperature storage may have been removed, at least in part, by the filtering process.

P73. Alanine-pyruvate conversions in bovine semen. R. J. FLIPSE AND W. R. ANDERSON, Pennsylvania Agricultural Experiment Station, University Park.

Previous work in this laboratory has shown that labeled glyoxylate is produced when spermatozoa are incubated with glycine-C^{14}. This has prompted investigation of the possibility that other transaminations may occur in these cells. A pyruvate-alanine system would be of particular interest. Lactic acid accumulates as a result of sperm metabolism, but the accumulation is less in glycine-containing diluents than in milk or egg yolk–citrate. Sperm extracts were prepared by mechanically disrupting the washed cells, centrifuging off cellular debris, and dialyzing the extract against phosphate buffer. Incubations were conducted at 37° C. with nitrogen as the gas phase. Each system included glycine, glyoxylate, pyridoxal phosphate, and *(a)* dialyzed sperm extract and alanine-1-C^{14}, *(b)* dialyzed sperm extract and pyruvate-2-C^{14}, *(c)* dialyzed seminal plasma and alanine-1-C^{14}, or *(d)* washed spermatozoa and alanine-1-C^{14}. In each of the systems tested, evidence was obtained of alanine-pyruvate conversion in excess of that obtained in the absence of extract or with heat-treated extract.

P74. Investigation of motility of sperm thawed at different temperatures and time intervals. H. H. BRUGMAN, M. E. POORE, AND R. P. SCHMIDT, University of Maine, Orono.

The Breeding Cooperatives in Maine, Massachusetts, and Connecticut supplied semen for this experiment. Semen was extended at the cooperatives in 20% egg yolk–citrate and 7% glycerol. Equilibration took place in transit to the Orono laboratory, and the time involved approximately 18 hr. Thirty-seven Holstein and Guernsey bulls were used. Two hundred and thirty samples from 3,160 ampules were analyzed. There was no significant difference in motility between thawing temperatures of 5 and 38° C. at intervals of 3, 6, 24, and 72 hr. Variation between studs was highly signifi-

cant ($P < 0.01$). Variation between breeds and between bulls within studs was also highly significant ($P < 0.01$).

P75. Recovery of sperm from the exteriorized bovine uterine cervix. H. D. HAFS, D. T. ARMSTRONG, AND R. W. BRATTON, Cornell University, Ithaca, New York.

The cervix of each of five heifer calves ranging in age from 1 to 21 wk. was relocated to the region of the left flank by a surgical technique similar to that used by Amantea in the dog and Reynolds in the rabbit. Following surgery, the heifers grew normally and there was no evidence of infection in the exteriorized cervix. Starting at 17 mo. of age, the mean length for 55 estrous cycles in four heifers was 20.3 days, with a standard deviation of 4.27 days, omitting one heifer which developed a cystic follicle. Ten billion nonmotile sperm were injected into the estrous uterus of each of the four heifers at each of nine successive estrous periods. After three incubation periods in each heifer, of either 0, 5, or 10 hr., an average of 49, 15, and 6%, respectively, of the injected sperm were recovered by flushing the uterus with two 250-ml. portions of saline. Three attempts to impregnate each heifer have failed. However, the relatively large number of sperm that may be recovered from the uterus by this surgical preparation offers an opportunity to study more extensively the effects of the uterine environment on sperm, particularly on their maturation.

P76. Effects of an intrauterine infusion of penicillin and streptomycin on reproductive performance in dairy cows. J. M. BUCKALEW AND J. A. ALFORD, Mississippi State College, State College.

For 2 yr., 89 animals have been given an intrauterine infusion of 500,000 units of penicillin and one gram of streptomycin in 20 ml. of sterile water during the estrous period prior to the one in which the cow was to be bred. A group of equal size served as a control. Cows that showed evidence of severe postpartum metritis, or that had a history of difficult breeding, were not used in the study. Bacterial swab samples were taken from the uterus during estrus and luteal phases prior to breeding, and during any subsequent estrous periods. To date, the treated cows have required 1.3 services per conception, as compared to 1.8 for the control group. Twenty-one of the control and five of the experimental animals have required three or more services to conceive. Bacterial examination revealed a wide variety of organisms present in the genital tract during all phases of the estrous cycle. No particular organism was found to be associated with the repeat-breeding cows, and no specific effect of the antibiotics on the uterine flora was revealed.

P77. Effects of oxytocin on bovine ovarian function. D. T. ARMSTRONG AND WILLIAM HANSEL, Cornell University, Ithaca, New York.

It has been shown that seven daily injections of oxytocin (free of contaminating gonadotropins), beginning on the day of estrus and prior to ovulation, consistently cause normally-cycling dairy heifers to return to heat and to ovulate from one to four days after injections are stopped (Armstrong and Hansel, *Fed. Proc.,* in press). Further studies have shown that even if oxytocin injections are not discontinued, heifers return to heat in approximately nine days afteh the previous estrus. This indicates that the return of estrus is not owing to oxytocin withdrawal, but more likely depends upon its action over a period of time. Four daily injections were insufficient to induce estrus. Gross and histological examinations of ovaries at various times during oxytocin treatment have revealed corpora lutea of smaller than normal size which, in some cases, contained fluid-filled cavities or cysts. The induction of estrus and ovulation by oxytocin could not be prevented by concurrent injections of prolactin, but could be prevented by the anticholinergic drug, atropine. These experiments are being continued, in an effort to elucidate the mechanism of this action of oxytocin and to determine if this method might be useful in controlling estrus and ovulation artificially.

P78. Morphogenesis and histochemistry of the bovine placenta between 16 and 33 days of gestation. J. S. GREENSTEIN, R. W. MURRAY, AND R. C. FOLEY, University of Massachusetts, Amherst.

Preliminary standards of daily development for the bovine placenta from 16 to 33 days of pregnancy have established, based on material recovered from 84 heifers and cows slaughtered at known intervals after insemination. The proliferating trophoblast gives rise rapidly to two distinct cell types characteristic of later stages: the ordinary trophoblastic columnar cells and the typical ruminant giant cells. The former appear to serve both a nutritive and secretory function. The latter are recognizable as early as 16–17 days, and their histochemistry suggests that they may play a role in the maintenance of the corpus luteum of pregnancy. The placenta contains only minimal amounts of glycogen, is rich in neutral fats, and a carbohydrate-protein complex is concentrated in the giant cell cytoplasm. The temporary yolk sac and vitelline circulation are gradually reduced in size, and function with the emergence on day 20 of the expanding allantois, resulting in the formation of a vascularized chorioallantois by day 27. Chorionic villous projections appear on day 31, preparatory to placentation. The successful development of the yolk sac, giant cells, vitelline circulation, and chorioallantois are all considered to be critical stages for embryo survival.

P79. Survival of rabbit ova stored at—79° C. MANSUR FERDOWS, C. L. MOORE, AND A. E. DRACY, South Dakota Agricultural Experiment Station, Brookings.

Fertilized rabbit ova were stored at —79° C. The eggs were equilibrated at 10-min. intervals with a one-fifth volume of 20% glycerol saline solution, so that a 10% glycerol solution resulted and was stored in a refrigerator until the temperature dropped to 2° C. The first temperature drop from 2 to —15° C. was carried out at the rate of a one-degree drop in temperature per each 10 min. The second step, from —15 to —35° C., was carried out at the rate of a one-degree drop in temperature per each 5 min. From —35 to —79° C., the temperature drop was one degree per each 2.5 min. At the end of this time, the ova were stored in a dry-ice chest until ready for transplantation. The ova were thawed at room temperature and transplanted into a doe, previously prepared for conception either by the injection of gonadotropin or by copulation with a vasectomized male. Sixty-five ova were transplanted into eight does. Of the eight does, two showed pregnancy—one with eight fetuses and one with ten developed fetuses—thus suggesting the possibility of storing ova at —79° C.

P80. Relationships among duration of estrus, ovulation time, time of service, and fertility of dairy cattle in Louisiana. J. G. HALL, C. BRANTON, E. J. STONE, AND R. B. LANK, Louisiana State University, Baton Rouge.

Studies of duration of estrus, ovulation time, time of service, and fertility in the L.S.U. dairy herd have revealed that recommendations to inseminators and farmers should be different from those for the temperate regions of the country. The average duration of estrus for 1,055 periods on 242 cows was 12.37 hr., ranging from 2 to 40 hr. During 10.05% of these periods, the cows failed to show standing heat. There was no apparent seasonal variation in duration of estrus. Little variation was found among the Holstein-Friesians, Jersey and Sindhi-Holstein crossbreds. The average number of days to first postpartum estrus was 31.95. The average interval between estrous cycles was 20.97 days. Only a limited number of cows were palpated to determine ovulation time. These data gave an average of 12.80 hr. from the end of estrus. These determinations are being continued. Conception rates for cows bred at 0–6, 7–12, 13–18, and 19–24 hr. after the first sign of estrus were 37.5, 58.5, 40.2, and 34.8%, respectively. Conception rate is being defined as diagnosed pregnancies to the first three services. There has been a total of 250 services to date.

P81. Bactericidal activity of inflammatory exudes from rabbit uteri. H. W. HAWK AND J. F. SYKES, Dairy Cattle Research Branch, USDA, Beltsville, Maryland.

Bactericidal substances in inflammatory exudate may play a part in the endocrine-controlled uterine defense mechanism. Exudate from estrous rabbit uteri inoculated 4 hr. previously is highly bactericidal, and few bacteria remain alive in the uteri; pseudopregnant rabbit exudate is only slightly bactericidal at 4 hr., and the uteri contain many bacteria at various intervals after inoculation. This could be owing to a continued lack of bactericidal substances in pseudopregnant rabbit uteri. Therefore, a test was made of the bactericidal activity of exudates at a later stage. Each uterine horn of eight pseudopregnant and eight estrous rabbits was inoculated with *Escherichia coli*. Exudate was collected from one horn 16 hr. later; the number of live bacteria was determined in the other. Leukocyte-free exudates and saline were inoculated with equal numbers of *E. coli* and incubated at 37° C. for 2 hr.; mean numbers still alive were: pseudopregnant rabbit exudate, 2,750; estrous rabbit exudate, 2,450,000, and saline, 117,000,000. Exudate of pseudopregnant rabbits is thus extremely bactericidal at 16 hr., even more so than estrous rabbit exudate ($P < 0.01$); despite this, more *E. coli* were alive in the pseudopregnant than in estrous rabbits. Factors other than a lack of bactericidal substances in pseudopregnant rabbit uteri must account for this difference.

P82. Effect of synthalin A on renal function in dairy bull calves. R. R. ANDERSON AND J. P. MIXNER, New Jersey Agricultural Experiment Station, Sussex.

Synthalin A is a toxic, hypoglycemic agent which has been reported to cause histological changes in the liver, kidneys, and alpha cells of the Isles of Langerhan, and also to depress hepatic function in dairy bull calves. Five Holstein bull calves (eight days of age) were injected intramuscularly with Synthalin A at the rate of 2.2 mg. per kg. of body weight. Plasma glucose levels decreased from a mean preinjection level of 85.6 to 32.8 mg. % 16 hr. after injection, and returned to normal levels again within 66 hr. after injection. Renal function was measured by the phenolsulfonphtalein (PSP) fractional clearance test. PSP fractional clearance decreased from a mean preinjection level of 0.0273 to 0.0102 within 16 hr. after injection (37.4% of normal), and returned to 81.0% of normal within seven days. The effects of Synthalin A in decreasing both plasma levels of glucose and PSP fractional clearance were highly significant ($P < 0.01$). Recovery of renal function was somewhat more delayed than the return to normal of plasma glucose levels, following the Synthalin A injections.

P83. Relationships between plasma protein-bound iodine and certain measures of reproductive and lactational performance in dairy cattle. H. D. LENNON, JR., AND J. P. MIXNER, New Jersey Agricultural Experiment Station, Sussex.

Plasma protein-bound iodine values were obtained at 60 days postpartum on 93 Holstein cows (139 lactations) and 34 Guernsey cows (57 lactations), over a 3-year period, and these values were correlated on a within-lactation basis, with certain concurrent measures of reproductive and lactational performance. Measures of reproductive performance included length of interval from parturition to conception, length of interval from first breeding to conception, and number of services per conception. Measures of lactational performance included 240-day 4% FCM production, maximum initial milk yield, persistency index, per cent fat, and per cent solids-not-fat. The Holstein data gave significant or highly significant negative correlations between the levels of plasma protein-bound iodine and the interval from parturition to conception (-0.255^{**}), interval from first breeding to conception (-0.251^{**}), services per conception (-0.213^{*}), and per cent fat (-0.179^{*}). No other correlations in either breed reached the level of statistical significance.

P84. Effect of pregnant mare serum on the sexually immature bovine testis.* ENRIQUE SANTAMARINA AND R. P. REECE, New Jersey Agricultural Experiment Station, New Brunswick.

Data are not available concerning the effect of gonadotrophic hormones on the testis of immature bovines; furthermore, hormonal therapy in the bull is based mainly on the limited information obtained from clinical data. An attempt has been made, by a study of the histological elements of the testis, to determine at what age the bovine testis becomes responsive to endogenous gonadotrophic hormones. Pregnant mare serum was administered to nine Jersey and Brown Swiss bull calves. Hormonal treatment was started when the calves were from 12 to 93 days of age. The total dosage ranged from 800 to 25,600 I.U. The diameter of the seminiferous tubules, the differentiation of the germinal cells, spermatogenesis and, especially, the maturation of the interstitial tissue were stimulated in some degree in every case. The interstitial tissue, however, was more sensitive to PMS than the other histological elements of the testis.

* Supported by a grant from the U. S. Public Health Service (RG—4726).

P85. Some causes of variation in estrual cycle length in Holstein-Friesian heifers. S. E. MARES, A. C. MENGE, W. J. TYLER, AND L. E. CASIDA, University of Wisconsin, Madison.

Effects of inbreeding, season of birth, infertile mating, and individuality have been determined on the lengths of 957 estrual cycles of 221 Holstein-Friesian heifers. Infertile inseminations were made at the beginning of 191 of the cycles and they will be referred to as return-intervals. The remaining estrual cycles had a mode of 21 days and a range of from three to 102 daays. The corresponding values for the return intervals were 20 and 4 to 92 days, respectively. There was a higher percentage of return intervals than of estrual cycles in the 24–33 day range and a lower percentage in the 19–23 day range. The intra-cow correlation for estrual cycle lengths (transformed to logarithms) within sire-lines and systems of mating was 0.096 ($P < 0.01$), and for return interval lengths, 0.062 ($P > 0.05$). Variances within cows differed among the different sire-lines, but did not differ significantly between outbred and inbred heifers, whether from outbred or inbred parents. Length of estrual cycles was affected by both season of birth and system of mating. The longest cycles were shown by spring-born, inbred heifers from inbred parents.

P86. Age at puberty in Holstein-Friesian heifers. A. C. MENGE, S. E. MARES, AND L. E. CASIDA, University of Wisconsin, Madison.

Effects of system of mating, growth rate, season of birth, and calfhood scours on age at puberty were studied in six sire-lines involving 184 heifers. Each sire-line was represented by outbred heifers from outbred parents, inbred heifers from outbred parents, inbred heifers from inbred parents, and outbred heifers from inbred parents (line cross). Significant differences existed among the sire-lines for age at puberty. System of mating also had a significant effect on age at puberty, with line-cross heifers attaining puberty at an earlier age than the inbreds from outbred parents ($P < 0.01$).

Six-month weight was 38 lb. less in heifers which had scoured as calves, compared to the nonscouring heifers ($P < 0.01$). Age at puberty had regressions of -0.77 days on 6-mo. weight and -0.36 days on weight gain from 6 to 12 mo. ($P < 0.01$ and $P < 0.05$, respectively). Scouring caused a delay of puberty only in the fall-born heifers. This delay of 136 days ($P < 0.01$) could not be accounted for by the effect of scouring through growth rate alone.

P87. Genetic improvement of dairy cattle attributable to sires used in artificial insemination in North Carolina. W. L. TUCKER AND B. R. FARTHING, North Carolina State College, Raleigh.

This study was initiated to evaluate the genetic advantage which actually has been realized in North Carolina from sires used in artificial insemination. The milk production, fat production, and fat test of 6,988 2×, mature

equivalent records of Holstein, Guernsey, and Jersey cattle were studied. The effect of season of calving on production was pronounced. Cows freshening during the months of December through May produced 21.0 lb. of fat and 595.0 lb. of milk more than cows freshening in June through November. First lactations of artificially sired daughters were compared with the first lactations of naturally sired animals freshening in the same herd, season, and year. The artificially sired daughters produced 15.7 lb. of fat and 366.0 lb. of milk more than their naturally sired contemporaries. The bias introduced by the daughter-dam comparison and the contemporary comparison, using all naturally sired contemporary records, was studied. Environmental differences, as well as selection of dams, biased the results from the daughter-dam comparison. When all naturally sired contemporary records were used for the contemporary comparison, the environmental bias was eliminated, but selection of animals which produced later records biased the results.

P88. Withdrawn.

P89. Heritability and repeatability of some blood constituents of dairy cattle. MOGENS PLUM AND A. B. SCHULTZE, University of Nebraska, Lincoln.

Blood samples of 50 heifers were analyzed at monthly intervals over a 10-mo. period beginning at 3 mo. of age. The blood samples were analyzed for hemoglobin, red blood cell count, circulating eosinophils, and blood sugar. The 50 heifers were sired by 12 different sires. Using averages of ten determinations, the heritability estimates obtained by paternal half-sister correlations were hemoglobin, 0.5, eosinophils, 0.6, red blood cell count, 0.7, and blood sugar, 0. The repeatabilities of determinations were as follows: blood sugar, 0, red blood cells and eosinophils, 0.1, and hemoglobin, 0.2. Heritability estimates based on single determinations are considerably lower than the ones determined by averages of ten analyses, viz., 0.20 for red blood cell count and 0.14 for hemoglobin and eosinophils.

P90. Comparison of a commercial milk-replacer ration with whole milk in a calf-raising system. M. W. NEATHERY, M. E. MCCULLOUGH, O. E. SELL, AND L. R. SISK, Georgia Mountain Experiment Station, Blairsville.

Thirty, four-day-old Guernsey calves were assigned equally, according to birth weight, to the following five rations: 1, whole milk; 2, milk replacer; 3, whole milk plus vitamin B_{12}; 4, milk replacer plus vitamin B_{12}, and 5, equal mixture of milk replacer and skimmilk. Liquid rations were fed daily at the rate of 10% of birth weight from four to 42 days of age. The carry-over effect of these rations was measured for an additional 42 days and a grain mixture and orchard grass hay were fed throughout the experiment. Weight gains from the whole milk rations (1 and 3) were significantly greater than gains from the milk-replacer rations (2, 4, and 5). Performance of both types of rations was increased, but not significantly, when vitamin B_{12} was added. Addition of skimmilk to the milk-replacer ration (2) did not significantly increase weight gains. The calves that received the whole milk rations continued to grow at a significantly higher rate during the carry-over period. Feed efficiency was significantly greater for calves receiving the whole milk rations. Some loss of hair was observed on two-thirds of the calves on the milk replacer ration (2). Scours was more prevalent among calves on the milk-replacer rations.

P91. Comparison of alfalfa hay, alfalfa silage, and corn silage as sole roughages for young dairy calves. K. E. GARDNER, University of Illinois, Urbana.

Twenty-one new-born calves were raised to 16 wk. of age on ad libitum feeding of one of three roughages—alfalfa hay, alfalfa silage, or corn silage. A total of 350 lb. whole milk was fed and a 19% T.P. starter was fed ad lib. up to 4 lb. daily. Gains averaged 154, 146, and 148 lb. daily on the alfalfa hay, alfalfa silage, and corn silage groups, respectively. When the effect of birth-weight differences was considered, the differences in gains were not significant. Average consumption of roughage was 179, 317, and 353 lb. for the three groups as listed. Starter intake was 275, 293, and 259 lb., respectively.

P92. Utilization of several fats by dairy calves. D. T. HOPKINS, R. G. WARNER, AND J. K. LOOSLI, Cornell University, Ithaca, New York.

As part of a study to determine satisfactory methods of adding fat to milk replacers, the following experiment was conducted: Different fats were mixed, at a level of 20%, with a basal diet of spray-dried skimmilk, Vitamin A, irradiated yeast, and Aurofac D. These diets were mixed with ten times their weight of warm water and fed to male Holstein calves. The fats used were (1) tallow (M.P. 40.0 C°), (2) cocoanut fat (M.P. 24.4 C°), (3) butter (M.P. 30.7° C.), (4) tallow and crude soybean lecithin, and (5) cocoanut fat and crude soybean lecithin. In addition, at the time of feeding, a homogenate of cocoanut fat, crude soybean lecithin, and water was added to the basal diet (6). Between the ages of 2 and 4 wk. digestion trials were conducted, with a minimum of four trials per diet. Feed was analyzed for ether extract; feces were analyzed for ether extract and soaps. The following digestion coefficients for total fat were obtained: (1) 59.2, (2) 65.4, (3) 67.3, (4) 72.1, (5) 95.6, and (6) 94.9. The coefficients for (5) and (6) were

significantly higher than those for the other diets. That for (4) was significantly higher than (1).

P93. Tetraalkylammonium stearate in a milk replacer and a dry calf starter. W. A. OLSON AND J. B. WILLIAMS, University of Minnesota, St. Paul.

Twenty-four calves were used in a 2^2 factorial experiment, to study the effects of tetraalkylammonium stearate in *(1)* a reconstituted dry skimmilk ration and *(2)* a calf starter containing 20% ground alfalfa hay, 25% ground corn, 20% soybean oil meal, 15% wheat bran, 12.5% ground beet pulp, and 2.5% vitamin and mineral supplement. Tetraalkylammonium stearate was added to the milk replacer to supply 200 mg. per calf per day, and to the calf starter at the rate of 40 mg. per pound for those animals receiving the treatment. Two Jersey bulls, six Holstein heifers, and 16 Holstein bull calves were employed from the fifth through the 42nd day of age. Whole milk was fed as one-half of the milk ration until 11 days of age. Spray-process nonfat dry milk was reconstituted 1:10 with water and fed at approximately 10% of the calves' body weight daily. The milk replacer was fed at 100° F. in open-top pails. The calf starter was offered ad libitum to the calves after the fourth day of age. Water was available to the calves at all times. Weight gains were similar with all treatments. Occurrence of scouring was greater and starter consumption was not as great, with the animals treated with tetraalkylammonium stearate, in both milk replacer and starter. There was no great difference in calf body-temperature between groups for the first 2 wk. of life.

P94. Effects of feeding volatile fatty acids to young calves. W. G. MARTIN, H. A. RAMSEY, G. MATRONE, AND G. H. WISE, North Carolina State College, Raleigh.

Nine calves (three replications), starting at three days of age, were fed the following diets for 12 wk.: Diet I: casein, fat, glucose, starch, and chopped hay; Diet II: casein, fat, glucose, and salts (31.9% of diet) of acetic, propionic, and butyric acids; Diet III: whole milk. Average daily gains (lb.) were: I, 0.79; II, 0.93, and III, 1.15. Samples of venous blood (0, 3, and 6 hr. after feeding) and of urine were taken weekly and analyzed for glucose, ketones, and volatile fatty acids (VFA). After feeding, blood glucose increased sharply for Diet III but decreased slightly for I and II. Conversely, blood ketones and VFA increased markedly for Diets I and II but did not change for III. For Diet II, blood VFA were highest at 3 hr. after feeding, and by 3 wk. of age, such values were similar to those found in the adult ruminant. Only small differences among diets were noted for any of the blood constituents immediately before feeding. At 0-hour, blood glucose tended to decrease with increasing age, whereas VFA and ketones exhibited no marked change. Calves on Diet II excreted the largest quantities of urinary ketones and VFA, whereas the least quantities of urinary glucose were excreted on Diet I. In general, the total excretion of all urinary constituents tended to increase with increasing age.

P95. Effects of acetate supplementation in calf feeding. J. R. STAUBUS, R. E. BROWN, C. L. DAVIS, AND W. O. NELSON, University of Illinois, Urbana.

In a preliminary study, six Holstein bull calves were fed milk at the rate of 10% of body weight, plus starter free choice, to 12 wk. of age. In addition to the basal ration, three of the calves were fed 50 g. of sodium acetate daily in the milk. The average blood glucose concentrations at 12 wk. were 95.3 and 89.7 mg/100 ml., as compared to initial levels of 98.1 and 110.6 mg/100 ml. for the acetate-fed and control calves, respectively. It is of interest that no pronounced drop in blood glucose occurred in either group. Acetate supplementation did not result in increased blood VFA levels. Rumen development as measured by wet weight, ratio of rumen and reticulum to abomasum, and papillary development varied between calves but not between groups. The most marked effect of acetate supplementation was in rate of growth and feed efficiency. The acetate-supplemented calves grew at a rate of 1.90 lb/day with a feed efficiency of 1.47 lb. D.M/lb of gain. The respective values for the control calves were 1.45 lb/day and 1.61 lb. D.M/lb of gain. Data from a more extensive trial involving 18 calves will be presented.

P96. Effect of a mixture of chlortetracycline, bacitracin, and penicillin on dairy calves. L. L. RUSOFF AND W. J. MILSTEAD, Louisiana Agricultural Experiment Station, Baton Rouge.

Twenty-four male Holstein calves were divided at random into three groups: Group I served as the control; Group II received 50 mg. of a mixture of three antibiotics (25 mg. of CTC, 18.75 mg. of zinc bacitracin, and 6.25 mg. of procain penicillin) daily, and Group III received 50 mg. of chlortetracycline daily. Colostrum was fed for the first three days, then whole milk at the rate of 10% of body weight up to 30 days of age. A plant-protein calf starter and medium-quality hay were fed beginning at four days of age. The calf starter for Group II was supplemented with a 1% supplement mixture of the three antibiotics (0.92 g. CTC, 0.72 g. bacitracin, and 0.24 g. of penicillin), and the calf starter for Group III, a 1% supplement of CTC (1.86 g. CTC per lb.). At 8 wk. of age, both antibiotic-fed groups (II and III) showed increased gains in weight by 15%, increases in height at withers, and increased

feed consumption over those of the control group. Hay consumption by the antibiotic-fed groups was started earlier and was almost double that of the control group. The few cases of scours present occurred only among the calves in the control group. Results at 12 wk. of age will be presented.

P97. Utilization of urea by young dairy calves as affected by aureomycin. L. D. Brown, D. R. Jacobson, J. P. Everett, D. M. Seath, and J. W. Rust, University of Kentucky, Lexington.

In an 84-day experiment utilizing 48 two-day-old Holstein and Jersey calves, a calf starter made up of 0.3 ground, medium-quality timothy hay, corn, oats, molasses, corn starch, and urea, and containing 12.1% protein equivalent (46.3% of N supplied by urea), was found to be adequate for above-normal growth of dairy calves. Limited milk was fed to 42 days of age. No long hay was fed. Starters One through Four, containing 6.5, 9.4, 12.1 and 15.3% protein equivalent, led to average daily gains of 0.54, 1.02, 1.29, and 1.19 lb., respectively. Apparent digestibility of crude protein and dry matter, as well as nitrogen retention, were significantly lower for Group One than for the other groups. Nitrogen retention increased significantly with age. Blood urea and plasma protein was significantly higher at two days of age than at 4, 8, or 12 wk. of age. Blood urea for Group Four was significantly higher than for Groups One and Two. Feed consumption and efficiency values present trends very similar to average daily gain. Aureomycin fed to half of the calves significantly affected average daily gain, heart girth, and feed efficiency.

P98. Digestive enzyme activity in the young calf. J. T. Huber, P. A. Hartman, N. L. Jacobson, and R. S. Allen, Iowa State College, Ames.

Twelve male calves were divided into two comparable groups at birth. One calf in each group was sacrificed at 1 day and at 1, 2, 3, 5, and 6 wk. Fourteen hours prior to sacrifice, calves in Group I received a test meal of whole milk plus 3% lactose, and Group II received whole milk plus 2.5% sucrose and 0.5% starch. Except for calves sacrificed at one day, diets similar to the respective test meals were fed from four days of age to sacrifice (colostrum, first three days). Gastric pepsin; pancreatic protease, lipase, and amylase, and intestinal lactase, maltase, and sucrase activities in the respective tissues were estimated. Test-meal residues in segments of the gastrointestinal tract were analyzed to determine nutrient digestion. Lactase decreased with age and, except for animals sacrificed at one day, was less in the posterior one-third of the small intestine. Maltase did not change with age or diet; no sucrase was detected. Pepsin was highest at 1 wk. and decreased thereafter. Lipase, pancreatic protease, and amylase increased during the first week. Carbohydrate digestion was 91 and 76% for Groups I and II, respectively. Scouring was greater in Group II. Average pH of the contents of the large intestine was 6.80 for Group I and 5.56 for Group II.

P99. Effects of the duration of whole milk feeding on growth of dairy calves. R. A. Ackerman, R. L. Steele, and H. O. Henderson, West Virginia University, Morgantown.

Two groups of 26 dairy calves of various breeds were fed and managed the same, except for the duration of whole-milk feeding, to determine if differences could be detected in growth and digestive disorders. One group of calves received 399 lb. of whole milk per calf from birth until they were 8 wk. of age; the other group received the same amount of milk per calf, but it was fed until the calves were 12 wk. of age. Hay and a starter were fed, free choice, through 16 wk., then the concentrates were limited and changed to a simple grain mixture. The results reported are on pooled data from two trials (the second trial is still in progress). The data were analyzed by analysis of variance, using the method of fitting constants. The first year's results show that the group fed milk for 12 wk. average 30 lb. heavier than the group fed the milk for only 8 wk., which approaches statistical significance. The 12-wk. group also required somewhat less TDN per pound of gain. Both groups were above normal in growth and remained in excellent health.

P100. Value of pasture for young dairy calves. M. C. Stillions, C. H. Noller, and B. W. Crowl, Purdue University, Lafayette, Indiana.

Twenty-two Holstein calves were assigned at random to three experimental groups: Group 1, control (barn-fed); Group 2, alfalfa-ladino-brome pasture, and Group 3, same as Group 2 plus hay free-choice. The calves were started on experiment as early as three days of age and on pasture between eight and 14 days of age. Limited whole milk was fed to 49 days of age. The calves consumed an average of 64.5, 43.4, and 36.9 lb. grain, and 44.5, 0.0, and 10.1 lb. of hay, respectively, during the first 60 days. The average daily gains were 0.97, 1.14, and 1.10 lb., respectively. The differences in average daily gains were not statistically significant. From 60 to 116 days of age the calves in the respective groups consumed an average of 187.3, 110.0, and 107.9 lb. grain and 256.2, 0.0, and 20.5 lb. hay, with an average daliy gain of 1.46, 1.67, and 1.70 lb. The differences in average daily gains were not statistically significant. Fecal egg counts for parasites were negative throughout the experiment. Observations suggest that the use of hay is instrumental in improving fecal firmness and the general appearance of young calves on pasture.

P101. Development of rumination in the young calf. E. W. SWANSON AND J. D. HARRIS, JR., Department of Dairying, University of Tennessee, Knoxville.

Observations of calf activity were made at 5-min. intervals for 24-hr. periods every 2 wk. until calves were 16 wk. old. A total of 16 Jerseys and 16 Holsteins was used. Four of the Jerseys observed and 13 other Jerseys were killed at from 2 to 3 wk. of age, to check the gastrointestinal contents. The calves were kept in individual pens. They were fed milk and high milk-content replacer at from 10 to 15% of body weight daily for 6 wk. Pelleted calf starter, chopped mixed hay, and water were available at all times after three days of age. At six to 14 days of age, 79% of the Holsteins and 64% of the Jerseys were ruminating. By 20 to 28 days, all of the Holsteins and 91% of the Jerseys were ruminating. During the 34- to 42-day observation period, the Holsteins ruminated 4.6 hr. and the Jerseys 5.0 hr. per day. In this same time (24 hr.), the number of separate rumination periods averaged 11.7 for Holsteins and 12.6 for Jerseys, with little subsequent increase until the 16 wk. of age. Examination of rumen contents showed that 11 calves killed at from 14 to 22 days had been ruminating normally, and six killed at from 12 to 15 days of age had not been ruminating effectively. These data indicate that effective rumination begins in the hand-fed calf before it is 3 wk. of age.

P102. Magnesium ammonium phosphate precipitated in rumen fluid and the relationship to frothy bloat. R. A. PHELPS, C. K. SMITH, AND C. F. HUFFMAN, Michigan State University, East Lansing.

Eight rumen-fistulated dairy cows, six of which were sets of identical-twins, were fed either two froth-producing or five nonfroth-producing rations. Rumen ingesta from these animals was centrifuged at an RCF of 30,000 and the supernatant adjusted to pH 9 with 10% NH_4OH. The precipitate obtained was qualitatively and quantitatively analyzed. Elemental, flame, and infra-red spectrographic analyses revealed mainly $MgNH_4PO_4 \cdot 6H_2O$ in the rumen fluid precipitate. The quantitative determinations revealed an average of five times more $MgNH_4PO_4$ from the froth-producing rations than from the nonfroth-producing rations. The quantity of $MgNH_4PO_4$ formed appeared to be directly proportional to the rumen-ingesta acidity. The amount precipitated was greatly stimulated by feeding small amounts of corn, but was not directly dependent on the amount of NH_4OH added, or on the apparent Mg and P intake of the animals. A correlated in vitro study of foam stability revealed a stable foam in rumen fluid below pH 4.5 and an unstable foam from 7.0. The entire study is being continued and additional data will be presented.

P103. Effect of a scabrous and a nonscabrous diet on rumen motility and intrarumen pressure. H. W. COLVIN, JR., S. D. MUSGRAVE, AND G. F. WILLIAMS. Oklahoma State University, Stillwater.

Four Ayrshire bulls were fed a nonscabrous diet consisting of a concentrate mix and a roughage in the form of a wood cellulose product, Solka-Floc BW-20, from 6 to 41 mo. of age. In 19 rumen motility trials, conducted when the bulls were 35 mo. old, it was found that the average fasting rumen contraction frequency per minute, primary contraction amplitude (cm. water), and intrarumen pressure (cm. water) were 2.1, 9.1, and 1.8, respectively. Not only were the bulls bloated during the motility trials but also before this, and up to the time scabrous material was included in the diet. When the bulls were 41 mo. old, the Solka-Floc BW-20 in the diet was replaced by prairie hay. Within 4 wk., the average fasting rumen contraction frequency per minute, primary contraction amplitude (cm. water), and intrarumen pressure (cm. water) were 1.6. 12.2, and −4.9, respectively. These values are within the normal range for cattle. The results suggest that scabrous material in the diet is necessary to maintain the tonic activity of the central pool of motor neurons responsible for rumen motility and, perhaps, eructation.

P104. Effects of constant environmental temperature (50 and 80° F.) on growth responses of Brown Swiss, Holstein, and Jersey calves. H. D. JOHNSON, C. S. CHENG, AND A. C. RAGSDALE, University of Missouri, Columbia.

Two groups of dairy calves (three each of the Brown Swiss, Holstein, and Jersey breeds) were subjected to constant environmental temperatures (50 and 80° F.) from 1 to 12 mo. of age. Body weight, heart-girth and wither-height measurements indicate that Holstein and Jersey calves generally did better at 50 than at 80° F.; whereas, Brown Swiss at 80° F. made better gains than at 50° F., as indicated by the following breed averages at 12 mo.: Body weight (Kg) Brown Swiss, 325.50; Holstein, 360.33; Jersey, 219.67 at 50° F.; and 334, 325.67, and 217.17, respectively, at 80° F. Conformation measurements suggest a greater ratio wither-height:chest-girth at 80 than at 50° F. in all breeds. Mature weight (Kg) predicted with the aid of modified exponential equation $W=A-Be^{kt}$ are Brown Swiss, 700, Holstein, 700, Jersey, 460 at 50° F.; corresponding values at 80° F. are 650, 660, and 440 kg. Percentage monthly declines in instantaneous growth at 50° F. are Brown Swiss, 5.9; Holstein, 6.2; Jersey, 5.7; and at 80° F., Brown Swiss, 6.3; Holstein, 5.8, and Jersey, 5.5.

P105. Effect of rising environmental temperatures (35–95° F.) on thyroid I^{131} activity of Holstein, Brown Swiss, and Jersey heifers.

H. D. JOHNSON, University of Missouri, Columbia.

Rising environmental temperatures depressed dramatically the in vivo thyroid I^{131} release rate of dairy heifers. Two groups of dairy calves (three of each breed—Holstein, Brown Swiss, and Jersey) weer raised from 1 to 12 mo. of age at constant 50 and 80° F. temperatures. Following the growth experiment, the heifers were exposed progressively (2- to 3-wk. periods) from 35 to 95° F. Relative humidity was maintained constantly at approximately 60%. Average K values of thyroid I^{131} activity at 35° F. for Holstein, Brown Swiss, and Jerseys raised at 50° F. were 0.10, 0.09, and 0.147, respectively, and 0.105, 0.139, and 0.171, respectively, for 80° F.-reared heifers. At 90° F., these values declined to 0.041, 0.049, and 0.084 for the 50° F. Holstein, Brown Swiss, and Jersey heifers, and to 0.053, 0.045, and 0.050, respectively, for the 80° F. heifers. Adaptation was evident in the above data, as the 80° F. heifers (those raised at 80° F.) showed a greater I^{131} thyroid activity at the lower temperatures (below 80° F.) than the animals raised at 50° F. (Supported by AEC.)

P106. Sex differences in certain bovine blood characters. A. B. SCHULTZE, M. PLUM, M. G. A. RUMMERY, University of Nebraska, Lincoln.

Erythrocyte, leucocyte, and eosinophil counts, as well as hematocrit, glucose, and hemoglobin values, of the blood of six Holstein heifer and six Holstein bull calves were determined at irregular intervals during the first year of life. There appeared to be no significant differences between the sexes in eosinophil or leucocyte counts. Erythrocyte count, hematocrit, and hemoglobin values were nonsignificantly higher in the blood of heifer calves up to about 6 mo. of age. After 6 mo. and up to 1 yr. of age these blood constituents were significantly higher in the bull calves. From 6 mo. to 1 yr. of life, the hemoglobin level of the males averaged 1.3 gm/100 ml. higher; the erythrocyte count, 1.7 million per $mm.^3$ higher, and the hematocrit 5.2% higher than the same constituents in heifers at corresponding ages. Glucose levels in male calves appeared to decline after birth less rapidly than they did in heifer calves, although the differences between sexes in rate of decline with age were not statistically significant.

P107. Studies of growth in Holstein, Ayrshire, Guernsey, and Jersey females as determined by: (1) Comparison of measurements at several ages to birth measurements as an index; (2) comparison of measurements of other breeds with Holsteins as an index. H. P. DAVIS AND I. L. HATHAWAY, University of Nebraska, Lincoln.

Data from 20 yr. of monthly body measurements of growth have been assembled, for three measurements each of heights, lengths, and widths and two each for depths and girths and also for weight. Breed comparisons for each measurement in terms of birth as an index are shown in charts for ages 1 through 5 yr. A composite of 13 linear measurements at 5 yr. showed the following increases in size: Holsteins, 2.57; Ayrshire, 2.53; Guernseys, 2.55, and Jerseys, 2.66 times birth measurements. The data show how animals increase body size during the first 5 yr. of life. Charts show the relative position in terms of Holsteins as a standard, for each of the other breeds, for weight and the 13 linear measurements for the first 5 yr. of growth. Each chart is a composite of 13 age measurement relationships from birth to 5 yr. Each type of linear measurement is also composited. Considering a composite of all 13 measurement relationships: Holsteins were 100; Ayrshires, 93.7; Guernseys, 92.0, and Jerseys, 88.1%.

P108. Heritability of gestation length in dairy cattle. J. C. DEFRIES, R. W. TOUCHBERRY, AND R. L. HAYS, University of Illinois, Urbana.

The objective of the present study was to determine what fraction of the variance of gestation period is genetic and, thus, to indicate the possibilities of shortening gestation length. From 2,063 gestation periods of the five dairy breeds in the University of Illinois herd, the within-breed, service-sire component of variance accounted for 10.5% of the total variance after adjusting for sex of the calf; thus, heritability was found to be 0.42. From the within-breed regression of offspring of dams involving 1,125 pairs, heritability was found to be 0.47. The difference of 0.05 between these two estimates could be caused by sampling errors or by the additive genetic effect of the dam on gestation length. The repeatability of gestation periods of the same cow was 0.20. These estimates of heritability indicate that the gestation length of dairy cattle may be shortened by selection, but experimentation is necessary to determine if any deleterious effects would be associated with this change.

P109. Relative merits of five ways of adjusting for the herd influence on artificially sired (AI) daughters' records. S. N. GAUNT, University of Massachusetts, Amherst.

Previous research at this station gave estimates of the intra-sire regression of the AI progeny record on each of the following contemporary averages: *(1)* The annual actual DHIA herd average, and stable-mates' average for *(2)* annual ME lactation, *(3)* 6-mo. ME season, *(4)* 7-mo. running ME season, and *(5)* annual actual age group. The average estimated regressions for milk and fat (ranging from 0.84 to 0.98) were used to adjust 4,590 and 1,404 AI progeny records of 296 and 145

Holstein and Guernsey sires for each contemporary average as: Daughter—b (herd or stablemate average—AI breed average). A variance component analysis (pooled data) of the adjusted records gave estimates of the herd component of 422, 123, 193, 111, and 308 for the respective measures (1, 2, 3, 4, and 5), as compared to 3.779 for *(6)* the adjusted record. Sire component estimates for *(6)* and *(5)* were highest, apparently due to inclusion of year effects, and in *(5)*, possibly, failure to remove fully sire-progeny age differences. Sire-herd component estimates were negligible. Further testing of these measures, using later proofs of these sires, is in process.

P110. Preliminary repeatability estimates of per cent total protein and other constituents in milk. S. D. Musgrave, R. E. Walton, F. A. Graybill, R. MacVicar, and J. B. Mickle, Oklahoma State University, Stillwater.

Data presented herein are based on 77 lactations from 63 Holstein cows. Daily composite samples of milk of individual cows were taken at monthly intervals. An average of 9.6 samples was obtained per lactation. Fourteen cows had two lactations. All lactations were initiated during the years 1953–1954. Repeatability estimates were computed by the usual least-squares procedure. When a single average value was used to represent each lactation, repeatability estimates were obtained as follows: *(a)* 0.2346 for per cent total protein (Kjeldahl method); *(b)* 0.0795 for per cent fat (Mojonnier method); *(c)* 0.5397 for per cent TS (Mojonnier method); *(d)* 0.4241 for per cent TS (Cenco procedure); *(e)* 0.6711 for per cent SNF difference from Mojonnier), and *(f)* 0.3227 for pounds of milk produced per sampling day. When individual monthly values were used to evaluate repeatability within lactations, then estimates were obtained as follows: *(a)* 0.1233 for per cent total protein; *(b)* 0.1261 for per cent fat; *(c)* 0.0967 for per cent TS (Mojonnier); *(d)* 0.1872 for per cent TS (Cenco); *(e)* 0.2188 for per cent SNF (difference from Mojonnier), and *(f)* 0.2360 for pounds of milk per sampling day.

P111. Error in estimation of lactational yields of solids-not-fat in milk from sampling one day a month. N. R. Thompson and G. C. Graf, Virginia Polytechnic Institute, Blacksburg.

Samples of individual milkings from 12 Holstein-Friesian cows were taken twice a day throughout the lactation period, and combined to make one-day composite samples. Percentage of butterfat was determiner by the Babcock method. Percentage of total solids was determined through use of the Watson lactometer and formula. Percentage of solids-not-fat was obtained by difference. Lactation yields of milk, butterfat, total solids, and solids-not-fat were obtained both as the sums of actual daily yields and as the sums of sampling-day yields, multiplied by days in testing periods. The high, low, and unweighted mean percentage deviations of calculated lactational yields from actual were: Milk, +1.2, −4.6, −2.3; butterfat, +4.1, −5.1, −1.6; total solids, +1.4, −4.1, −2.0, and solids-not-fat, +1.5, −4.8, −2.4.

P112. Genetic variation in clinical mastitis. C. W. Young, J. E. Legates, and J. G. Lecce, North Carolina State College, Raleigh.

The heritability of clinical mastitis was investigated where quantitative scores for clinical mastitis were determined on a lactation basis. Estimates were obtained from 416 complete lactations on 285 cows in four state-owned North Carolina herds. Relative efficiencies of selection based on indexes including laboratory findings, in addition to clinical mastitis and clinical mastitis alone, were compared. Also studied was the relationship of clinical mastitis to udder height. The heritability estimates obtained from the data were 0.71 from the paternal-sister correlation, and 0.21 from the daughter-dam regression. In addition, repeatability estimates of 0.35 and 0.40 were obtained. From these estimates, and additional information on components of hereditary variance, a value of 0.24 was arrived at as a reasonable value for the heritability of clinical mastitis. The inclusion of bacteriological data and leucocyte counts from monthly milk samples in the selection criteria resulted in only a 0–5% increase in efficiency of selection against clinical mastitis. The correlation between udder height and clinical mastitis was −0.27 ($P < .01$) when herd and breed differences were removed, and −0.14 ($P < .05$) when additional differences owing to age were removed.

P113. Heritability and repeatability of milk-yield records of Holstein-Friesian cows in Louisiana with and without certain environmental corrections. C. Branton, E. J. Stone, and G. D. Miller, Louisiana State University, Baton Rouge.

Considerable research has been done on the environmental corrections of milk-production records in the temperate regions of the world. However, very little has been done in the subtropical areas, such as the Gulf Coast region of this country. Therefore, such studies were initiated on the records in the L.S.U. Holstein-Friesian herd. Age-correction factors were derived by the multiplicative method, from data on 1,837 lactations of 406 cows during the period 1927–1956. It was found that the current breed factors underconvert the records of young cows by a considerable amount. Body weight was much more highly correlated with milk yield than was age at calving. Studies of the year and season-of-freshening effects on 722 lactations of 283 cows, during the period

1947–1957, revealed that both had highly significant effects on milk yield. At present, the heritability and repeatability estimates on these records from daughters of 31 bulls are being calculated on the records, before and after appropriate corrections for the effects of age at calving years and seasons of freshening. Daughter-dam comparisons on these bulls also are being considered.

P114. Estimates of environmental trends and biases resulting from errors in age factors and repeatability. C. R. HENDERSON, Cornell University, Ithaca, N. Y.

Unbiased evaluation of noncontemporaries from records requires estimation of intra-herd environmental trends and consequent adjustment of records. The author presented a method for doing this at the 1949 meeting of this Association. The required computations are laborious; consequently, it was not practical at that time to try the method on many herds. Recently, these computations have been programmed for the IBM 650, and a study has been made of 1950–1954 records in 11 New York herds. Using New York age factors and repeatability $=0.40$, the environmental change was estimated to have caused an average decline per year of 3.0 lb. fat per cow. Since the method requires age adjustments and a choice of repeatability, the effect of errors in these factors on the estimation of environmental trend was investigated. For each pound too much by which first-lactation records are built up, the environmental trend is biased downward by 0.22 lb. per cow per year. For each 0.01 by which the repeatability actually used in the estimation procedure exceeds true repeatability, the down bias is 0.08 lb. fat per cow per year.

P115. Analyses of feed efficiency on six sire groups in the Beltsville Holstein herd. N. W. HOOVEN, JR., AND C. A. MATTHEWS, Dairy Cattle Research Branch, Beltsville, Maryland.

One hundred and four cows by six sires were used in this study. Milk production and feed consumption data were compiled from the first lactation records on all cows. Second-lactation records were available and used on 66 cows, representing three of the six sire groups. Feed efficiency as used in this study was defined as:

$$\frac{\text{lb. of FCM}}{\text{lb. of TDN consumed}}$$

Analyses of variance indicated significant differences between sire groups with respect to feed efficiency. A high correlation was also found between feed efficiency and milk production. A covariance analysis was made, holding milk production constant. Results showed that significant differences remained between sire groups. This suggested that at least some of the genetic differences in feed efficiency are independent of milk yield. An intra-class correlation on 66 cows with first- and second-lactation records was made, to determine the repeatability of feed efficiency and FCM (ME). Results showed that the repeatability for feed efficiency was about two-thirds as high as for milk production.

P116. Relations between weight at first calving and milk production during the first lactation. R. H. MILLER AND L. D. MCGILLIARD, Michigan State University, East Lansing.

Michigan DHIA–IBM records for 4,677 Holsteins, 1,001 Guernseys, and 501 Jerseys were used to fit constants by least squares for the independent influences of age and weight on production in the first lactation. These effects indicated that delaying calving of heifers is economically disadvantageous, and that heavier heifers have little or no advantage over lighter heifers of similar age. Intraherd partial regressions were about 75 lb. of milk per month of age and 200 lb. of milk per 100 lb. weight at first calving. Large correlations between herd averages of weight and production contributed significantly to larger partial regressions of milk on weight, when herds were ignored. Genetic correlations between weight and production were of the order of 0.3. Heritabilities based on paternal-sib correlations ranged from 0.3 to 0.5 for weight and from 0.2 to 0.4 for production in the first lactation. Dairy heifers should be bred as soon as sufficiently large to minimize possible harmful effects on length of productive life. Selection for increased milk production may result in larger size. Correcting production for weight may remove some genetic variation in production.

P117. Relation between weight and production in dairy cattle. B. R. FARTHING AND J. E. LEGATES, North Carolina State College, Raleigh.

This study was initiated to determine the relative influence of live weight and age on production in Holstein and Jersey cows, and to develop a method of utilizing live weight as an aid to selection. The Holstein data consisted of 690 normal lactations and heart-girth measurements of 580 cows from ten North Carolina state institutional Holstein herds. One Jersey herd provided 396 normal lactations and weights of 175 cows. The weight measurements were taken the first month after calving. All lactations were expressed on a 305-day, 2× basis. The within-cow linear and quadratic effects of age and weight indicated that age is more effective than weight in accounting for variance in actual production. In the Jersey data, the intra-cow regression of mature equivalent (M.E.) production on live weight did not account for an appreciable portion of the variance in M.E. production. In the Holstein data, increases of 390 lb. in M.E. milk and 16 lb. in M.E. fat were associated with an average increase of 100

lb. in live weight. The greatest expected genetic improvement in M.E. milk was obtained when weight-corrected M.E. milk was used as the basis for selection.

P118. Relating age-conversion factors to herd level. S. R. SEARLE AND C. R. HENDERSON, Cornell University, Ithaca, New York.

In appraising animals on production records, those of young cows are usually adjusted by age-conversion factors. In no case have such factors been related to the level of the herd in which the cows make their records. This has been considered in a study of 20,432 cows in 749 Holstein, or predominantly Holstein, herds in New York State, in the four seasons, fall, 1950, through spring, 1952. For each herd-year-season, the cows younger than 5 yr. were divided into four groups, and regressions estimated of the difference between each age-group average and the 5- to 9.5-yr.-old average, on the herd average; they were found to be significantly different from zero. With these regressions, an age-corrected herd average can be calculated from the actual herd average, and the age-conversion factors for a herd are then based on the regression lines, using the age-corrected herd average. These regressions indicate that in high-producing herds the corrections for age should be greater than in low-producing herds. On the average, the resulting factors are close to the multiplicative ones now being used in New York State, suggesting that herd differences are largely, but not wholly, taken account of by such factors.

P119. Effect of season, age, and stage of lactation on type ratings of Holstein cows. C. J. WILCOX, K. O. PFAU, R. E. MATHER, AND J. W. BARTLETT, New Jersey Agricultural Experiment Station, Sussex.

Spring- and fall-type ratings by official inspectors from 1945 to 1957 were coded to maintain equal intervals (1.0) between ratings. Lactation number was used to represent age, with scores grouped into first lactation, second lactation, and third to fifth lactations (mature). Each lactation was divided into three periods, *(a)* parturition to 60 days, *(b)* 61 to 240 days, and *(c)* 241 days to next parturition. Only cows with five or more ratings were included in the study. Least-squares analyses, using dummy variables, yielded estimates of the various environmental effects independent of each other. Over-all ratings for fall were lower than spring by a coded value of 0.09 ($P<0.08$); first- and second-lactation scores were lower than mature scores by values of 0.52 and 0.29 ($P < 0.01$), respectively. Early and mid-lactation scores were similar, but late-lactation scores were 0.15 lower ($P < 0.01$). Subratings for fall, and for first and second lactations, were generally lower than their counterparts. Scores in late lactation were higher, except for dairy character, with scores of 0.19 lower ($P < 0.01$) than mid-lactation scores. These results indicated that adjustments for the significant influences would result in more accurate studies in this field.

P120. Association of certain type evaluations and type scores of first-lactation Holstein females. E. L. AKINS, T. M. LUDWICK, H. E. RICKARD, E. R. RADER, AND W. M. ETGEN, The Ohio Agricultural Experiment Station, Wooster.

Two hundred and seventy-three Holstein females and their 22 sires and dams (seven herds) were evaluated for type on a detailed score-card basis, to determine the relative influence of various type information in predicting the type of the daughter at the time of her first calf. Evaluations were made on the females at three early ages (3, 6, and 12 mo.), and 3 mo. after first-calving. Sires were evaluated only at maturity (5 yr.). Interpretation of the analyses indicates the following associations: *(a)* With the exception of the head-and-neck category, the breakdown classification of the sire had little value in predicting the type of his daughters; *(b)* no significant predictions for daughters' type could be made from information on the dam; *(c)* estimates of type-score repeatabilities ranged from 0.2 to 0.5 among younger ages (3 to 6, 3 to 12, 6 to 12 mo.); *(d)* repeatability of type scores from younger ages to after-calving was very low, *(e)* in general, all udder components had low repeatabilities and predictabilities.

P121. Heritability of various characteristics of body conformation and their association with production of Holstein-Friesian cows in Canada. D. G. GRIEVE, J. C. RENNIE, AND G. E. RAITHBY, Ontario Agricultural College, Guelph.

The official production and type classification records of 17,818 Holstein-Friesian cows distributed in 2,192 herds were used in this study. All records were completed during a 2-yr. period, 1953 to 1955. The production records were standardized, using two systems; namely, mature equivalent and breed class average. The intra-herd correlations between paternal half-sibs were used in estimating the heritability of the 18 different type characteristics listed under the sections on "Rump" and "Legs and Feet" on the official Holstein-Friesian Selective Registration score-card. These characteristics and the heritability estimates are as follows: High pelvis, 0.17; narrow rump, 0.11; low thurls, 0.14; low pins, 0.10; high tail-head, 0.10; coarse tail-head, 0.12; legs too straight, 0.09; sickled legs, 0.15; coarse legs, 0.16; close hocks, 0.13; thick thighs, 0.00; weak pasterns, 0.24; toes out (front), 0.15; toes out (rear), 0.13; shallow heel (front), 0.25; shallow heel (rear), 0.15; open-toed (front), 0.00, and open-toed (rear), 0.21. Phen-

otypic correlations and regressions between eight of the type characteristics and milk and butterfat production, calculated on an intra-herd and sire basis, were all small in magnitude. The correlations between these eight type characteristics and the percentage of butterfat were in all cases essentially zero.

P122. Contribution of breed of sire, breed of dam, and system of mating to rate of growth in crossbred as compared with purebred dairy cattle. D. C. SHREFFLER AND R. W. TOUCHBERRY, University of Illinois, Urbana.

Forty-three purebred Holsteins and Guernseys and 44 reciprocal Holstein-Guernsey crosses were compared, with respect to body weight, wither height, chest depth, heart girth, paunch girth, and lengths from withers to hips, hips to pinbones, withers to pinbones, and shoulder to pinbones. Measurements at 3, 6, 12, 18, 24, 30, 36, and 48 mo. of age were analyzed by the method of least squares; the normal equations were solved by using an inverse matrix. The effect of breed of dam was highly significant for all measurements at all ages, and in every instance was larger than the effect either of breed of sire or of mating system. The effect of breed of sire became significant in general at about 12 mo., but was not significant for the lengths from hips, withers, or shoulder to pinbones. The effect of mating system was not significant, but absence of nonadditive genetic effects associated with crossbreeding is not unexpected, when the relatively high heritability of thees measurements is considered.

P123. Effect of two systems of mating on butterfat production of six lines of Holstein-Friesian cattle. C. E. KRAEMER, W. J. TYLER, AND A. B. CHAPMAN, University of Wisconsin, Madison.

The data for this study were the first lactation butterfat records of 78 outbred ($F_x=0$) and 69 inbred ($F_x=0.13$ to 0.29) progeny in six sire lines of purebred Holstein-Friesian cattle at the Emmons Blaine, Jr. Experimental Farm, Lake Mills, Wisconsin. The lactations were begun over a 6-yr. period. After adjustment to a 2× M.E., 305-day basis, the data were fitted to a least-squares model, and constants were computed for the effects of year, line, and system of mating. A preliminary analysis showed that the interaction between years and lines within systems of mating was not significant. The analysis of variance showed that the differences among years and the interaction between lines and systems of mating were highly significant. The regressions of butterfat production on percentage of inbreeding for the six lines were −2.4, −2.4, −2.4, −2.7, −3.1, and −7.0 lb. Five of the six lines showed similar effects of inbreeding and it is apparent that one line contributed mainly to the interaction of lines and systems.

P124. Performance of artificially sired daughters and their contemporary naturally sired herd-mates in stratified levels of herd production. N. J. GUDERYON, E. L. CORLEY, AND J. W. DUCKWALL, University of Wisconsin, Madison.

The 2×, 305-day, M.E. production of artificially sired (AI) daughteers was compared with their contemporary naturally sired (NS) herd-mates on a within-herd, within-calving year basis. A total of 51,023 Holstein-Friesian and 13,725 Guernsey milk and butterfat records were studied, after adjustment for an unequal number of AI and NS lactations within herds and years. When all available records were used, AI-NS differences were +157 lb. milk and +9 lb. butterfat for Holsteins and −20 lb. milk and +2 lb. butterfat for Guernseys. When 11,945 first records were used, these differences were +163 ($P > .01$) and +8 ($P < .01$) for Holsteins and +89 ($P > .05$) and +4 ($P > .05$) for Guernseys. Herds represented by first records were stratified into low, medium, and high levels, according to actual yearly herd averages for butterfat yield. Holstein AI-NS differences were +10 ($P < .01$), +13 ($P < .01$), and +1 lb. of fat, and +225 ($P < .01$), +175 ($P < .05 > .01$), and +79 ($P > .05$) lb. of milk in low, medium, and high herds, respectively. Corresponding differences for Guernseys were 0, +7, and +5 lb. of fat and +79, +220, and −39 lb. of milk, none of which was significant. These data suggest that Holstein AI daughters are genetically superior to their NS herd-mates in low and average DHIA herds in Wisconsin.

P125. A method of determining adequate sample size to estimate heritability or repeatability from the analysis of variance. STANLEY WEARDEN, Kansas Agricultural Experiment Station, Manhattan.

Heritability *(h)* and repeatability *(r)* are often estimated by functions of an intra-class correlation obtained through the analysis of variance. In the completely random design under Model II, two variances are estimated: That among genetic groups (σ_g^2) and that within groups (σ_w^2). In repeatability, a genetic group consists of multiple records on the same individual. The ratio $\sigma_g^2/\sigma_w^2=\theta$ can be expressed as $P/(\frac{1}{a}-P)$, where P is the genetic parameter (h or r) and a is the coefficient of relationship among the individuals within a genetic group. An adequate sample is one containing a sufficient number of observations *(n)* per genetic group, to detect, with known probability, significant differences among groups when they exist. This concerns the power of the test $[\beta(\theta)]$, which is distributed according to the central F-distribution. By fixing the power, and guessing the minimum size of P, it is possible to solve the equation $n=\frac{1}{\theta}[F\beta/F_a)-1]$. $F\beta$ is the F-value corresponding to the power,

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P126. Genetic structure of dairy cattle herds in terms of additive and dominance relationships. A. E. FREEMAN AND C. R. HENDERSON, Cornell University, Ithaca, New York.

Data from 11 New York grade and registered Holstein herds were used to characterize the additive and dominance relationships within these herds. All possible additive relationships (a_{ij}) and the dominance relationship (d_{ij}) among non-inbred animals were computed, after tracing ancestry two generations beyond the foundation animals of each herd. Excepting ancestors of the foundation cows, the mean a_{ij} for all herds was 0.04. Distribution of these values showed that 73% of these animals were unrelated and 92% of the a_{ij}'s were less than 0.175. The mean coefficient of inbreeding was 0.007. The relationships among all non-inbred animals with a_{ij}'s > 0, whose complete records started between the ages of 22–34 mo., inclusive, were computed. The mean additive and dominance relationships were 0.13 and 0.007, respectively. For these animals, 77% of the a_{ij}'s were < 0.2 and 50% were < 0.1; also, 91% of the d_{ij}'s were < 0.025. These low relationships indicate that, regardless of the magnitude of the population dominance variance and the variance due to inter-allelic interactions involving both genes of at least one pair, the contribution of these components could, in the average, contribute very little to the total genetic covariance between relatives in a random-mating population.

EXTENSION SECTION

E1. A device to pipette samples directly into test bottles. W. R. DAVEY, The Pennsylvania State University, University Park.

Two studies were made, using procedures which should reduce the time and work involved in conventional D.H.I.A. sampling. The first study consisted of removing 8.8 ml. of milk from the thoroughly mixed milk, with a properly calibrated pipette, after each milking, and introducing it into a milk test bottle. The second study made use of the Cornwall Leur-Lok syringe with appropriate attachments. After adjusting delivery to 8.75 ml. of water, samples of nights' and mornings' milk were delivered to the test bottle, similarly to the method used in the first study. The data revealed that in 853 comparisons with the 8.8–ml. pipette, 86.6% of the tests fell within 0.1% of the control procedure and 97.6% checked within 0.2%. In the method using the syringe, 81.3% of the results fell within 0.1% of the control procedure and 96.3% checked within 0.2%, based on 621 comparative tests. The method would seem to provide an improvement over present procedures, resulting in less sample handling and les schance of erroneous results owing to souring or churning. However, this procedure would preclude aliquot sampling when the interval between milking caused extreme variations in milk weights.

E2. Results of accuracy studies on weighing and sampling devices. M. E. SENGER, North Carolina State College, Raleigh.

Continued expansion in production testing will require simple and convenient methods for weighing and sampling milk in pipeline systems. At the American Dairy Science Association meetings in June, 1957, weighing and sampling devices for use in the National Cooperative Dairy Herd Improvement Program were approved. Accuracy tolerances were suggested as a guide in determining which devices should be acceptable for standard DHIA work. With the increased popularity of pipeline milkers, many devices for weighing and sampling milk from individual cows are now available. However, some of these devices have not been adequately checked and tested for accuracy under proper and uniform conditions. Consequently, those supervising DHIA testing have not had the information needed to determine which devices are within the acceptable accuracy tolerances. A survey was made of extension dairymen supervising DHIA testing, to find out which devices have been adequately tested and checked for accuracy. A summary of this survey, and reports by several states on the results of acuracy studies on weighing and sampling devices, will be given.

E3. Motivation of supervisors and dairymen in identifying cows in Dairy Herd Improvement associations. L. H. RICH AND B. H. CRANDALL, Utah State Agricultural College, Logan.

The incentive for identifying large percentages of D.H.I. cow records is of interest, and prospective results indicate that this can be done with minimum effort. Mechanical processing gives legibility, accuracy, and completeness in reports, provided supervisor's monthly barn sheets arrive at computing service listing correct data. The supervisor's job then is largely completed. Cows once identified with acceptable requirements for sire provings carry such identified listings permanently, with changes in status, lactation data, etc. as accumulative periods occur. Supervisors (full- or part-time), carefully selected and rewarded for time expended; D.H.I. officers given responsibility; dairymen kept fully informed; national eartagging program with cooperating agencies, and computing service personnel showing affability, accuracy, dispatch, and informative attitudes are factors making for herd permanency and for obtaining more complete identifications. From 79 to 81% of 305-day records processed monthly were acceptable in 1957 for the National Sire Proving Program (sent in card-lots). Covering a 3-yr. period, data were processed,

providing listings for 1,323 sires and their respective daughters' records (by sire). Processing of mature equivalents monthly, for properly identified breeding records only, gives incentive for cooperation of dairymen and supervisors. Working materials, district schools, and close relationship of dairymen, supervisors, computing service and extension personnel, are paramount. Success is its own reward.

E4. Comparison of machine-calculated records and lactations submitted by D.H.I.A. fieldmen. A. G. SENDELBACH AND E. L. CORLEY, University of Wisconsin, Madison.

These data were taken from 43 standard D.H.I.A. Wisconsin herds in ten associations with 20 field supervisors. The 305-day lactation reports submitted routinely by supervisors were computed later with punch-card equipment. This provided an opportunity *(1)* to study the fieldmen's accuracy in computting records, and *(2)* to determine if production differences occur in reported and nonreported lactations. Over a 3-yr. period, 3,476 lactations were computed by machine, of which 2,926 (84%) were reported by fieldmen. Of these, 427 (15%) were in error as to calving date or cow identification. The remaining 2,499 were compared with the machine-computed records, regarding the frequency and percentage of error in milk and butterfat yield. Milk-fed errors in excess of 500 lb. totaled 162 (7%) of the reported records, whereas 134 (5%) were in error by 20 or more lb. of fat. The mean absolute error in milk yield was 117 lb. for all reported lactations and 575 lb. for the 508 records exceeding 1% in error. Reported records exceeded those not reported by 1,147 lb. milk, 60 lb. fat, and 32 days. After removing all records of less than 180 days, these differences were 730, 34, and 15.

E5. Comparison of age-conversion factors for Ayrshire butterfat records. R. D. CASSELL, R. S. DUNBAR, JR., R. G. MITCHELL, AND I. D. PORTERFIELD, West Virginia University, Morgantown.

Eighty thousand, four hundred and fifty-two Ayrshire butterfat production records, completed between January 1, 1946, and December 31, 1955, were used to develop additive and multiplicative age-conversion factors. The accuracy of these factors was checked by applying them to immature butterfat production records on cows that freshened at from 72 to 95 mo. of age during the period between January 1, 1956, and May 31, 1957.

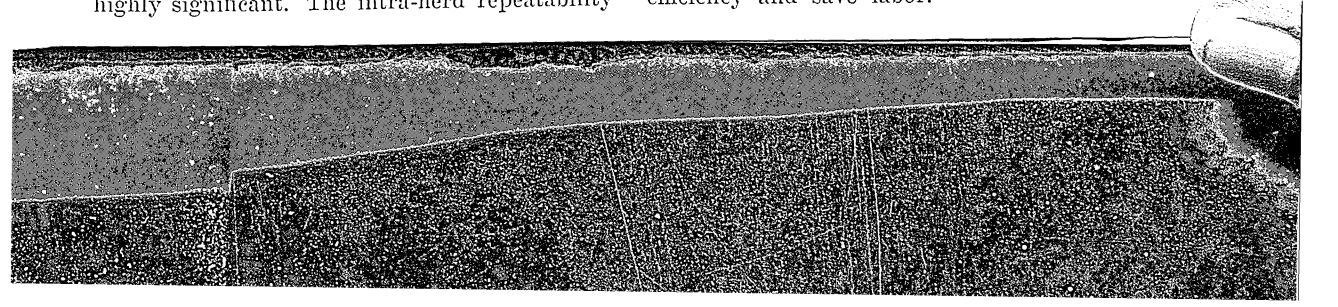
Intra-herd variances, repeatabilities, and correlations were used in comparing the present Ayrshire factors, the additive, and the multiplicative correction factors for predicting the animal's production at maturity. The variation among herds and among cows within herds was highly significant. The intra-herd repeatability value for the present Ayrshire factors was 0.474; the additive factors, 0.322, and the multiplicative factors, 0.471. Intra-herd correlations between adjusted immature and actual mature records for the three conversion factors were all significant at 2 and 3 yr. of age and highly significant at 4 and 5 yr. of age.

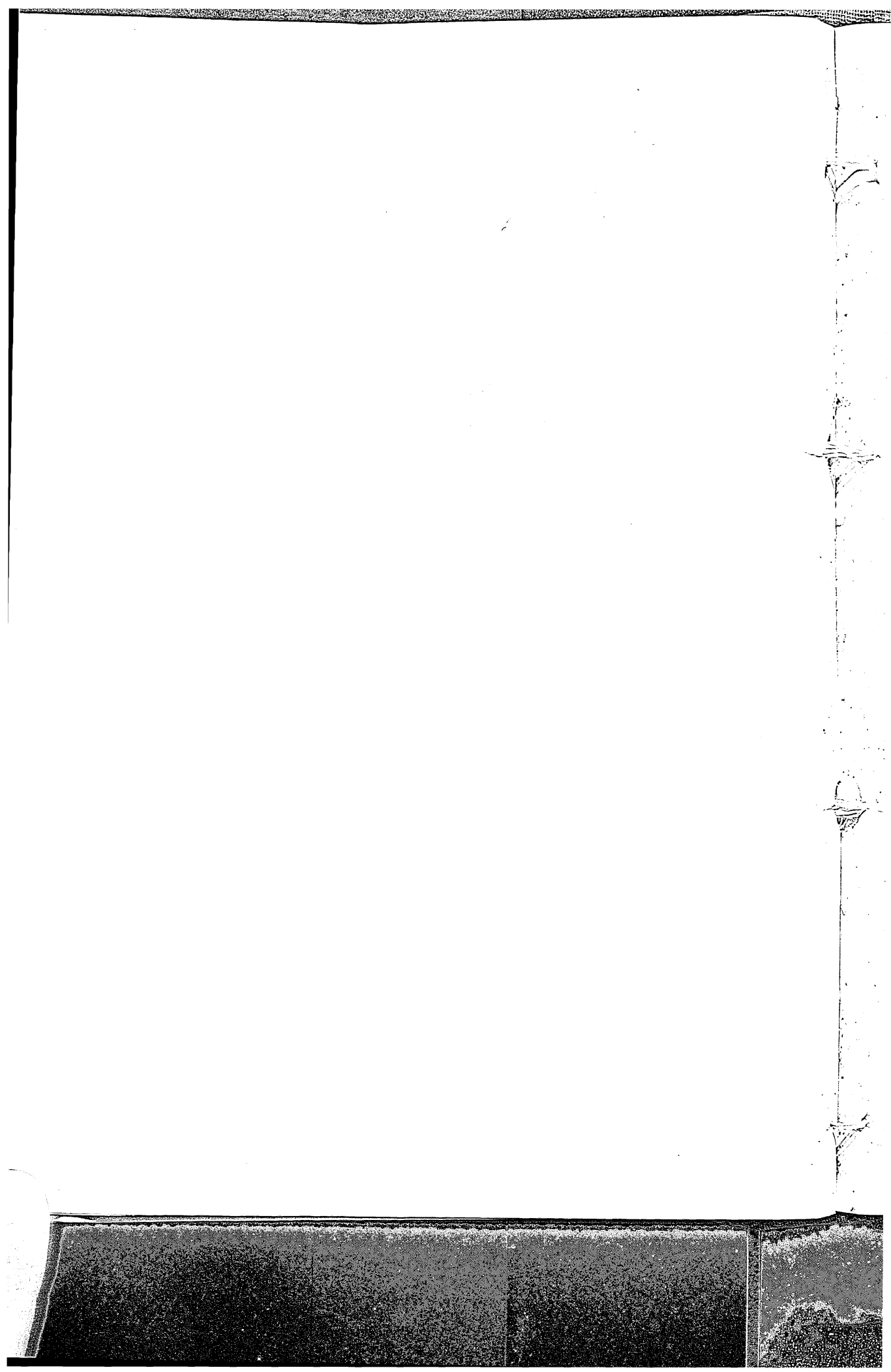
E6. Increasing efficiency on farms producing milk for manufacturing purposes. G. S. PARSONS, North Carolina State College, Raleigh.

In the last few years, many manufacturing milk producers with small investments have made their jobs easier, more pleasant, and more profitable. This has been done through the use of labor-saving devices, such as self-feeding of silage from horizontal silos, loose housing of cattle, ground-level hay storage, elevated-stall milking parlors, and the cow-to-can milking machine. Among the greatest labor-saving devices in the production of manufacturing milk have been the elevated stalls and the cow-to-can milker. The elevated-stall milking parlors are a simple arrangement in which the cows are elevated to allow milking without stooping. All milking operations, grain feeding, and the moving of cows in and out are done by one person, with little walking. Labor required for milking can be reduced one half. One person can easily milk 12–15 cows per hour. No elaborate building is needed. Almost every farm has a barn or other similar building in which a parlor can be installed. Through the use of these labor-saving devices, part of the family can work in industry and still maintain the dairy operation. Also, many physically handicapped people find that these arrangements enable them to continue milking when otherwise they could not.

E7. Managing large herds of 100 cows or more. W. R. VANSANT, University of Arizona, Tucson.

In 1952, it was observed that changes in herd management were taking place in Arizona. A yearly survey was conducted by DHIA supervisors and the following changes were revealed: increase in herd size, 30%; additional hired milkers, 19%; bulk-storage tanks, 58%; pipeline milkers, 34%; and decrease in raising replacements, 57%; raising hay, 25%; pasture, 27%. As a result of this study, an extension program was developed which included barn-layout designs, a herd-replacement program, artificial insemination, handling and treatment of individual animals, maternity quarters, feed storage, and many other recommendations to improve efficiency in herds of from 180 to 240 cows. More mechanization is required for large herds, such as crop-harvesting equipment, mechanical feeding wagons and trucks for soiling crops and for silage, special arrangements for feeding hay, use of mechanized devices for cleaning corrals, and many others to improve efficiency and save labor.





DISPERSIBILITY OF FOAM-DRIED FAT-CONTAINING MILK PRODUCTS

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SUMMARY

It was found that foam-dried whole milk could be stored for at least 1 yr. at 73° F. without losing its excellent dispersibility. However, some loss was found when the material was stored at 100° F. Forewarming at 190° F. for 2, 5, and 8½ min. caused no significant change in initial dispersibility, but after storage at 100° F. for 1 mo. the dispersibiltiy was found to vary inversely with the holding time used in forewarming. The foam-drying process was successfully extended to a lactose-enriched whole milk, an acid-modified whole milk, and a chocolate-flavored whole milk. Products of enhanced dispersibilty were prepared by this method when compared to commercially available materials of similar types.

The dispersibility of nonfat milk powders has not yet been matched commercially with dry whole milk. The fat content of whole milk, amounting to a minimum of 26% by weight of the dry product, poses many problems not found with dry nonfat milk. A foam-dried whole milk *(3)* possessing excellent dispersibility in ice water with spoon stirring has been produced successfully on a pilot plant scale in the Philadelphia Laboratory of the Eastern Utilization Research and Development Division of the Agricultural Research Service. The current paper discusses the effect of storage on the dispersibility of the foam-dried product made from flash-pasteurized whole milk, with and without additional heat treatment of the whole milk. The value of foam drying as a means for improving the dispersibility of other dry fat-containing milks is discussed.

RETENTION OF DISPERSIBILITY ON STORAGE

Storage tests were made on foam-dried whole milk containing 29.5% fat and 2½% moisture, crushed to pass through a 20-mesh screen, and packaged in cans under an atmosphere containing 98.5% N_2 and 1.5% O_2. The cans were stored at 38, 73, and 100° F. and evaluated for dispersibility after storage periods of 2 wk. and 1, 2, 4, 6, and 12 mo. Dispersibility rate curves *(3)* were used to evaluate the effect of storage on the dispersing property. The tests showed that the dispersibility of foam-dried flash-pasteurized whole milk remains unimpaired for at least 1 yr. at room temperature (see Table 1).

The reasons for this good dispersibility and its permanence are not fully understood. However, some of the contributing factors are as follows:

1. *Avoidance of protein alteration during processing.* That the proteins are not denatured is evidenced by ultracentrifugal tests [described in *(3)*] which show the whey proteins to have the same sedimentation pattern as those in fresh pasteurized milk (see Figure 1). As might be expected, the

Received for publication November 7, 1957.

[1] A laboratory of the Eastern Utilization Research and Development Division, USDA.

TABLE 1

Effect of storage at 73° F. for one year on dispersibility rate of foam-dried whole milk

Stirring time	Dispersibility rates	
	Fresh	Stored
(sec.)		
20	70.2	76.4
40	87.8	84.3
60	93.7	93.6
80	95.3	94.5
100	95.3	96.8
120	97.8	98.1
Mean dispersibilities	90.0	90.6

product contains only trace amounts of undispersed material by the ADMI solubility index test.

2. *Dispersion of the fat globules which remain discrete throughout processing and storage.* Microscopic examination of the fat in the concentrate before drying, and in the milk reconstituted from the dried stored product, shows no evidence of coalescing or clustering. The fat globules remain discrete and are of the same size as immediately after homogenization. They appear to be "bound," perhaps by physical-chemical means, within a matrix of readily dispersible material.
3. *The distinctive shape of the particles.* Unlike spray-dried particles, particles of crushed foam-dried milk are irregular in shape, have one very small dimension, are not powdery; hence, have little tendency to cake- or ball-up in contact with water.

Storage at 100° F. impaired the dispersibility (as shown in Figure 2). After 1 mo. of storage there was a loss of about 5% in dispersibility with 2 min. of

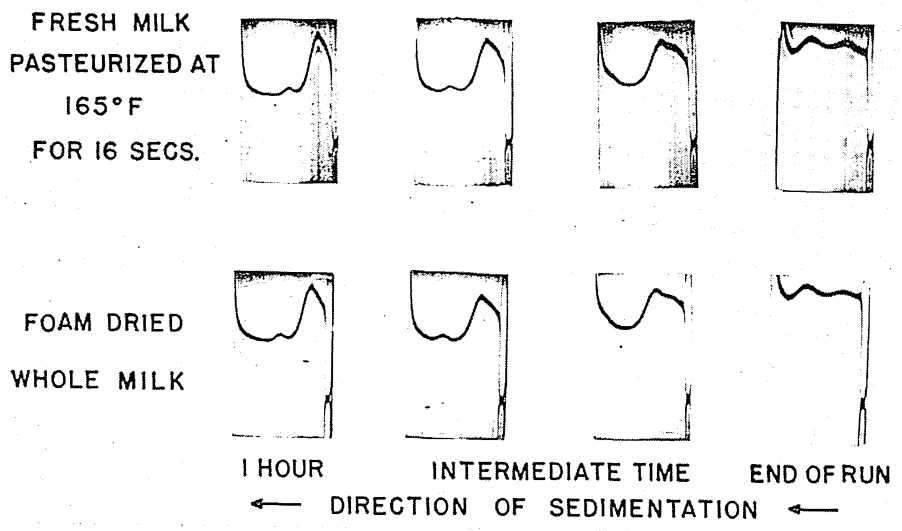


FIG. 1. Ultracentrifugal patterns of whey protein concentrates *(4)*.

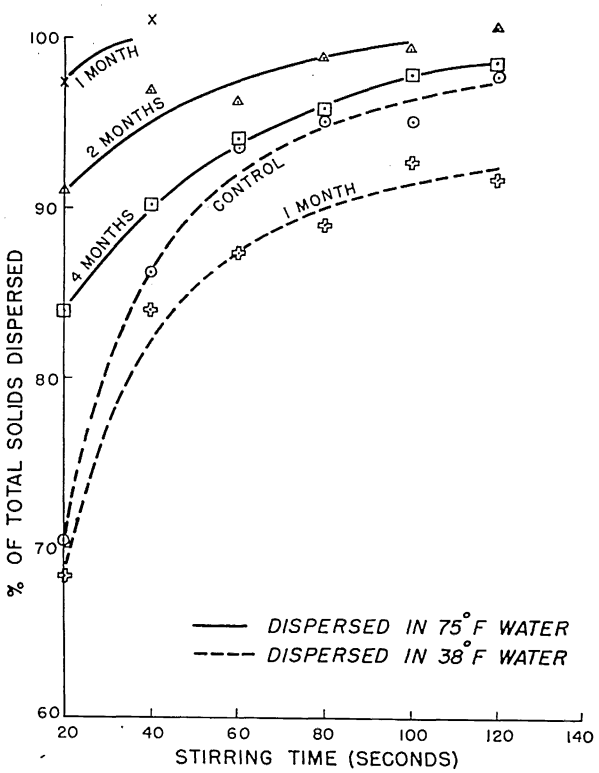


FIG. 2. Dispersibility rate changes with storage at 100° F.

stirring in 38° F. water. However, when 75° F. water was employed, which is the temperature normally used in dispersibility tests, complete dispersibility resulted after only 35 sec. of stirring. Even after 4 mo. of storage at 100° F., dispersibility in 75° F. water was excellent.

Microscopic examinations of the fresh and stored samples showed in both cases that the fat globules were imbedded in a milk solids-not-fat matrix. The only apparent change resulting from the storage was in this matrix. In the case of the stored sample, it disintegrated slowly in water. There was no evidence, however, of "fat migration," to which the well-known loss of dispersibility in some dry whole milks has been attributed. It appears probable that the loss in dispersibility is a consequence of protein insolubilization resulting from high-temperature storage.

EFFECT OF FOREWARMING

To determine the effect on dispersibility, heat treatments were given flash-pasteurized whole milk of 3.2% butterfat, using conditions favoring the development of sulfhydryl groups *(2)*. The milk, after deaeration, was quickly heated to 190° F. in a Votator[2] and held at that temperature for approximately 2, 5,

[2] Mention of companies and commercial products anywhere in this paper does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

and 8.5 min., respectively, then quickly cooled to below 100° F. These three batches, and a control given no heat treatment beyond that of flash pasteurization, were foam-dried by the published procedure *(3)*.

These were nitrogen-packed in cans with an oxygen content below 1.5% and stored at 100° F. for 1 mo. Dispersibilities were determined in 38° F. water with 60- and 100-sec. stirring. The results are shown (Table 2).

TABLE 2

Effect of forewarming of whole milk on its dispersibility after foam-drying[a]

Heat treatment		Dispersibility *(%)*[b]					
		60 sec. stirring			100 sec. stirring		
Time	Temp.	Fresh	Stored[c]	Loss	Fresh	Stored[c]	Loss
(min.)	*(°F.)*			*(%)*			*(%)*
0	.....	95.1	92.4	2.7	97.1	94.0	3.1
2.1	191	95.7	85.0	10.7	98.3	87.8	10.5
5.1	190	92.6	74.2	18.4	96.5	78.7	17.8
8.6	189	93.0	75.7	17.3	96.5	78.3	18.2

[a] All products contained 26% fat (MFB).
[b] Averages of data from four pilot-plant runs at each set of conditions.
[c] One month at 100° F.

It is apparent that the heat treatments had little effect on the dispersibility before storage. However, changes were induced which were responsible for a more rapid loss in dispersibility during storage than occurred in the product which had no heat treatment. The loss also increased with time of holding the heated milk. The losses in dispersibility were accompanied by a slight increase in insolubles suggesting that, as previously observed in the case of the unheated product stored at 100° F. (see Figure 2), loss in dispersibility was a consequence of protein insolubilization. It is important to avoid damage to the proteins if a dry *whole* milk of good dispersibility in ice water is to be produced. Damage may occur either during processing or on high-temperature storage of the dry product.

The importance of forewarming in improving the flavor stability of spray-dried whole milk is well known. The need of forewarming to protect against atmospheric oxidation during processing of foam-dried whole milk is unnecessary, because dehydration is carried out under high vacuum and low temperature, in contrast to the high-temperature oxidative conditions typically used in spray-drying. The latter conditions, coupled with the tenacious retention of entrapped oxygen within the spray-dried particle, create a different storage problem than is encountered with the foam-dried material. Moreover, preliminary tests using the forewarming conditions of Table 2 showed no improvement in the flavor stability of gas-packed, foam-dried material. The effects of forewarming and other factors on the flavor stability of the foam-dried product are currently under investigation.

The elimination of heat treatment (typically 180° F. for 30 min.) can pose a problem in controlling bacterial growth during operations. This can be over-

come by the sound practice of using milk of low bacterial count and by processing rapidly with only short-time holdup at points where temperature favors bacterial growth. For example, in the foam-drying process it was proposed to concentrate the milk at 90° F. *(3)*. If this were done in a conventional recirculation type evaporator, the relatively long time required to concentrate the milk would favor the growth of bacteria. Thus, this operation could better be carried out in a single-pass evaporator, wherein concentration is accomplished in a matter of seconds. This has the added advantage of permitting higher evaporation temperatures without protein denaturation. Evaporators of this type are characterized by the mechanical agitation of the heat-sensitive film on the heated surface. They are manufactured domestically by at least two companies. Another single-pass evaporator of possible use here is one developed at the Eastern Utilization Research and Development Division for fruit juices *(1)*.

Thus, the foam-drying process would be carried out somewhat as shown in Figure 3.

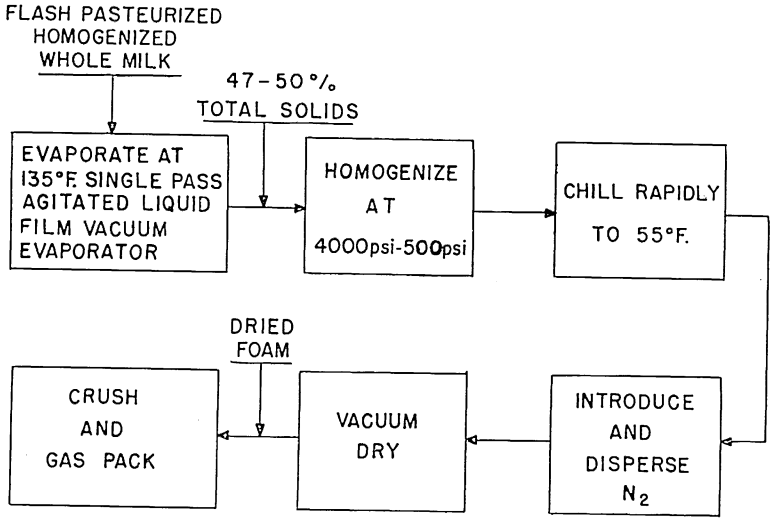


FIG. 3. Process for preparing foam-dried whole milk.

We have observed in the course of some 300 pilot-plant runs, drying whole milk, that when on rare occasions the solubility index was above about 0.1 cc., the otherwise excellent dispersibility of the dry whole milk was impaired. In contrast, it is well known that spray-dried *nonfat* milks of excellent dispersibility may contain 0.4 cc. insolubles or more. Similarly, in foam-dried nonfat milk, a higher level of insolubles can be tolerated without impairment of dispersibility than with dry whole milk. Therefore, the high-temperature processes commercially feasible with nonfat milk are at a disadvantage with whole milk.

Foam-drying of other fat-containing milks. Adaptation of the foam-drying process to fat-containing milks other than whole milk was shown to enhance the dispersibility of these products. They include a chocolate-flavored whole milk

and infant foods such as lactose-enriched whole milk and an acid-modified whole milk. Table 3 gives pertinent drying data on these products. Evaporation temperature and the temperature and pressures of homogenization were, in all cases, the same as in the standard process for whole milk.

TABLE 3

Conditions used in foam-drying fat-containing milk products

Material whole milk	Total solids in concentrate	Drying			Product moisture
		Time	Max. temp.	Volume increase	
	(%)	*(min.)*	*(°F.)*	*(fold)*	*(%)*
Chocolate-flavored whole milk	50.70	207	107	32	1.33
Lactose-enriched whole milk	55.38	161	106	24	1.80
Acid-modified whole milk	40.85	118	114	10	1.03

1. *Chocolate-flavored whole milk.* A commercially available chocolate-flavoring powder composed of Dutch-processed cocoa, sugar, salt, an emulsifier, and flavorings was added to whole milk of 4.1% butterfat, 12.9% total solids, in the ratio of 1 to 25.8 lb. The mixture was evaporated, homogenized, gassed, chilled, foam-dried, and crushed through a 20-mesh screen in the usual manner *(3)*.

2. *Lactose-enriched whole milk.* Lactose monohydrate was dissolved in whole milk of 4.1% fat, 12.9% total solids, in the ratio of 1 to 19 lb. This resulted in a dry product similar in composition to commercial products of this type. The formulation was then evaporated, gassed, chilled, foam-dried, and crushed through a 20-mesh screen in the usual manner *(3)*, except that evaporation was carried beyond 50% solids. This was possible because at 50% solids the concentrate showed no tendency to gel and was more fluid than whole milk at the same solids content.

3. *Acid-modified whole milk.* The dry product was prepared from the following recipe:

	(lb.)
Whole milk (4.1% fat, 13.0% solids)	41.24
Dextrins-maltose	1.21
Sucrose	1.21
Corn starch	0.83
Lactic Acid (85% soln.)	0.24

The resulting dried product was similar in composition to commercial products of this type. The milk was warmed to 90° F. and the ingredients added with stirring. Curdling occurred just before all of the acid had been added. In spite of this, the formula was processed in the usual manner *(3)*, except that evaporation was carried only to about 41% solids, due to the high viscosity of the concentrate at that point. A subsequent experiment showed that a solids

content of about 38% yields a foam of more desirable physical form than was obtained in the present instance.

Dispersibility of the dry materials. Figure 4 contains dispersibility rate curves for the three foam-dried products and, in the cases of the infant foods,

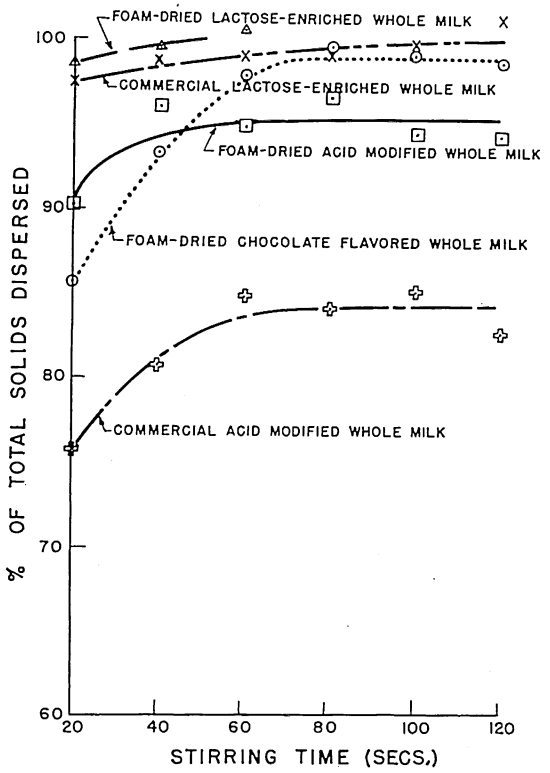


FIG. 4. Dispersibility rates of dry fat-containing milk products.

for commercially available products of similar compositions. These curves were obtained by use of the dispersibility rate test as previously published *(3)*, with the following exceptions:

1. The weight of the dry chocolate-flavored whole milk was increased from 15 to 17.4 g. per 90 cc. of water. This was done so that its dispersibility curve would be based on the solids content of a typical chocolate-flavored whole milk.
2. The infant foods were dispersed in water at 100° F. instead of the usual 38° F., because in household practice water at or above the former temperature would normally be used for reconstituting products of these types.

The curves show that foam-drying can be advantageously applied to these materials. The curves show that a dry chocolate-flavored whole milk of excellent dispersibility may be prepared by foam-drying. The results suggest the application of foam-drying to whole milk flavored with fruit juice concentrates, es-

sences, honey, or other natural flavors. The authors are currently investigating this.

ACKNOWLEDGMENT

The authors express their appreciation to R. E. Townend of the Eastern Utilization Research and Development Division for his ultracentrifugal studies and to R. W. Whittenberger of the Eastern Utilization Research and Development Division for his microscopic observations.

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RAPID DETERMINATION OF MILK SALTS AND IONS. I. DETERMINATION OF SODIUM, POTASSIUM, MAGNESIUM, AND CALCIUM BY FLAME SPECTROPHOTOMETRY

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SUMMARY

A rapid determination of milk cations is presented. A trained technician can make four to six complete analyses a day for sodium, potassium, calcium, and magnesium. The cation exchange resin Dowex 50 is used to eliminate, quantitatively, the total amount of cations from a milk serum prepared according to the procedure of Jenness *(2)* and Murthy and Whitney *(4)*. Then, the cations are quantitatively eluted with 3.5 *N* HCl and analyzed by flame spectrophotometry. The accuracy of the method is due to the fact that the analyzed solution does not contain other substances than the cations to be analyzed—hydrochloric acid and ammonia. The interference of phosphoric acid, especially, and of organic materials, is avoided.

Many attempts have been made to determine accurately and rapidly the mineral components of milk. However, it seemed difficult to apply one single method to the analysis of the four major cations in milk, sodium, potassium, magnesium, and calcium. Sutton and Almy *(7)* have used successfully the ion exchange chromatography technique to separate these four cations, with subsequent titration of the pure chlorides. This procedure includes ashing of the milk samples, chromatography, evaporation of excess HCl, and titration of chlorine; consequently, it is long and tedious. Keirs and Speck *(3)* described the widely used flame spectrophotometric technique of milk ash solutions, and they give a good over-all view of the problem. The paper of Riethmüller *(5)* is referred to for the fundamentals of emission spectrophotometry of the elements and for the most suitable equipment and techniques.

To avoid the time-consuming ashing of milk samples, the technique of Jenness *(2)*, modified by Murthy and Whitney *(4)*, was applied to get a casein-free milk serum containing all cations present in the milk. Jenness *(2)* was interested only in Mg and Ca analysis by the titration method of Schwarzenbach *et al. (6)*. Murthy and Whitney *(4)* used their milk serum for direct flame spectrophotometric analysis of Na, K, and Ca. Keirs and Speck *(3)* stated that the phosphoric ion interferes seriously with the Ca flame analysis. To overcome this difficulty, Murthy and Whitney *(4)* eliminated an important part of the interfering anions with anion exchange resins. The sample ready for analysis still contained large amounts of organic material, making it impossible to determine magnesium accurately.

The procedure described in this paper made it possible to analyze four to six milk samples a day with accuracy, and the same sample is used to analyze the four cations, Na, K, Mg, and Ca.

Received for publication December 18, 1957.

METHOD

Instrument. For preliminary trials, a Beckman B spectrophotometer with flame attachment has been used. The present study refers to data obtained with a Beckman DU spectrophotometer with flame attachment and photomultiplier. The instrument was operated with a hydrogen-oxygen burner as indicated in Table 1. The instrument allows accurate measurements with a maximum deviation of single determinations of ± 2%.

TABLE 1

Suitable conditions for flame spectrophotometric analysis of Na, K, Ca, and Mg

Analyzed element	Suitable concentration range	Wave length	Photo tube	Sensitivity		Slit width	Gas pressure	
				DU	Photomultiplier		H_2	O_2
	(p.p.m.)	*(mμ)*				*(mm.)*	*(p.s.i.)*	
Potassium	0 to 60	767	Red	0	..	0.01	2	10
Sodium	0 to 10	588	Blue	0	2	0.01	2	10
Calcium	0 to 60	554	Blue	0	3	0.04	2	10
Magnesium	10 to 60	371	Blue	0	3	0.20	4	15

Preparation of the milk serum. The procedure of Murthy and Whitney *(4)* with minor variations was used. To 60 ml. of fresh milk or 7.5 g. of dried whole milk, water is added to a volume of approximately 100 ml. The casein is precipitated by adding five ml. of 1 *N* HCl and the solution is allowed to stand for 10 min. The sample is adjusted with 1 *N* NH_4OH to pH 4.0–4.1 and the volume made up to 150 ml. The solution is filtered through an analytical-grade filter paper or centrifuged. A 100-ml. sample, equivalent to 40 ml. of fresh milk or to 5 g. of dried whole milk, is collected for analysis of the cations. The casein-free filtrate can be obtained, in variation of this procedure, by adding to the diluted milk 2 ml. of glacial acetic acid instead of 5 ml. of 1 *N* HCl. In this case, subsequent heating at 80° C. for 10 min. is necessary, but adjusting the pH with ammonia can be omitted. In this study, the HCl precipitation procedure was used.

Preparation of the pure chloride solution of cations. The 100-ml. sample of casein-free acid serum was passed quantitatively through a column of 10 ml. of Dowex 50 cation exchange resin in the hydrogen form. We used uniform particle-size of the resin passing through 30-mesh screen (wet) and retained on a 40-mesh screen. The columns were 30 cm. long, with an inner diameter of 0.9 cm., and were closed with a thin-tipped stopcock, and the resin was retained by a small plug of glass wool just above the stopcock. The sample was passed through the column at the rate of 4 ml. per minute, then the resin was washed with three portions of 10 ml. of distilled water until the pH of the effluent was neutral.

During the adsorption cycle of the cations, the absence of cations in the effluent solution is checked by the flame test, with a drop of the solution taken from the bottom of the column with a platinum wire. Since the cation-binding capacity

of the Dowex 50 column should be about double the cations to be adsorbed, no cations should be detectable during adsorption, including the washing with water.

By this procedure, all anions and all organic substances are eliminated from the sample to be analyzed. The cations are eluted with 75 ml. of 3.5 *N* HCl at a rate of 2 ml. per minute. The first 3 ml. of the effluent, consisting of water only, are discarded. The following 50 ml. of acid solution, which contains the cations (Figure 1), are collected in a 50-ml. volumetric flask. The end of the elution is verified by the flame test, with a platinum wire. Traces of calcium still may be present after 50 ml. of acid has passed through the column, but are without significance for the quantitative analysis of Ca.

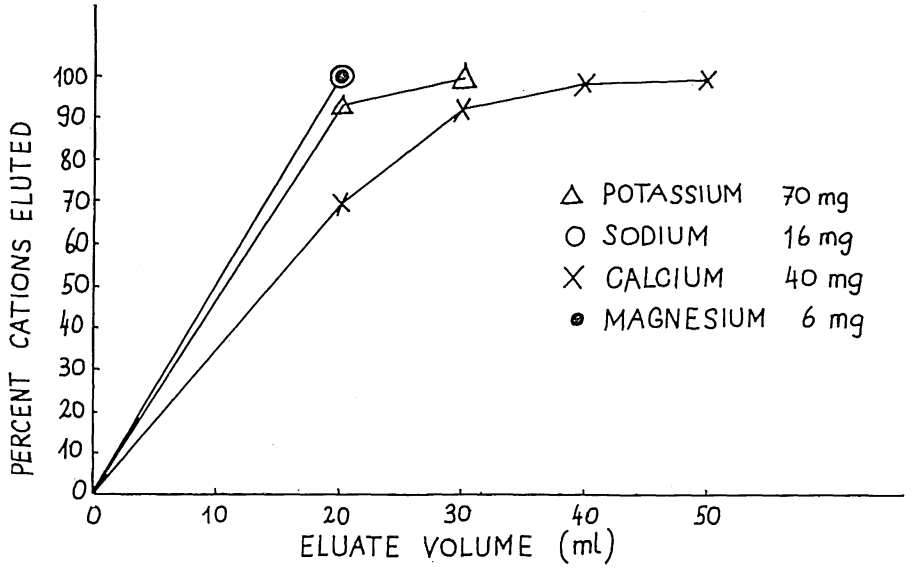


FIG. 1. Elution with 3.5 *N* HCl of potassium, sodium, calcium and magnesium, adsorbed on 10 ml. of Dowex 50 cation exchange resin.

The columns are then ready for a new adsorption cycle, after the complete elution of the cations with 3.5 *N* HCl and after rinsing the column with distilled water to eliminate the concentrated acid.

This procedure is to some extent similar to the method described by Denson *(1)*, who eliminated the phosphoric acid from solutions of dissolved ashes derived from biological material by adsorbing the cations on an Amberlite IR-105 cation exchange resin. (Denson reported that he also had trouble with the elution of calcium.)

Flame photometric determination of Na, K, Mg, and Ca. The hydrochloric acid solutions of the cations are thoroughly mixed and diluted to appropriate concentrations. For Na and K analysis, a dilution of 1:100 in 0.5 *N* HCl is suitable, yielding concentrations of from 1 to 10 p.p.m. of Na and of from 3 to 60 p.p.m. of K (Figures 2 and 3). For Ca analysis, a dilution of 1:25 in 0.5 *N*

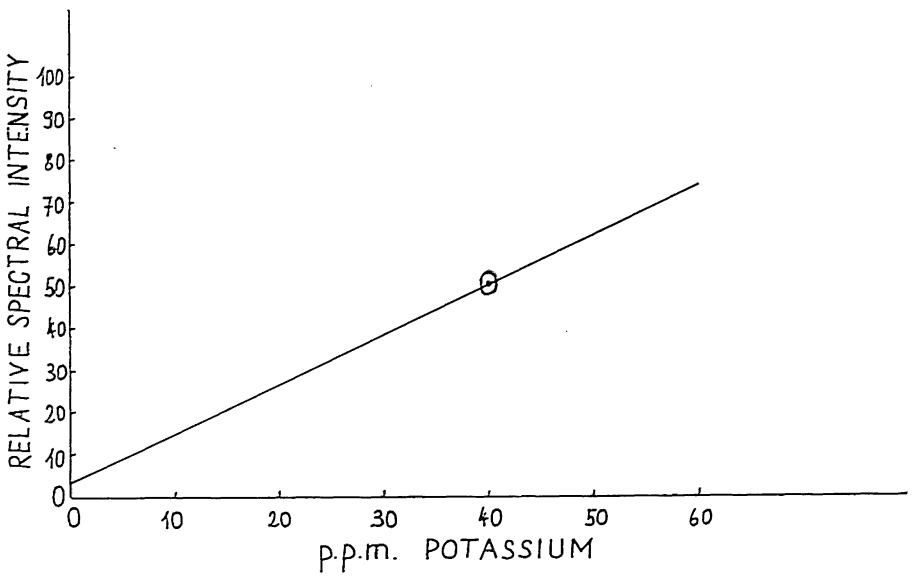


FIG. 2. Calibration curve for potassium. The curve is obtained by measuring the potassium emission (Table 1) of 12 concentrations of K ranging from 5 to 60 p.p.m., added to a standard solution containing 3.5 p.p.m. of Na, 8 p.p.m. of Ca, 1.2 p.p.m. of Mg, and 14 p.p.m. of NH_4OH in 0.5 *N* HCl.

⊙ = reference point arbitrarily chosen to check the photometer before running an analysis.

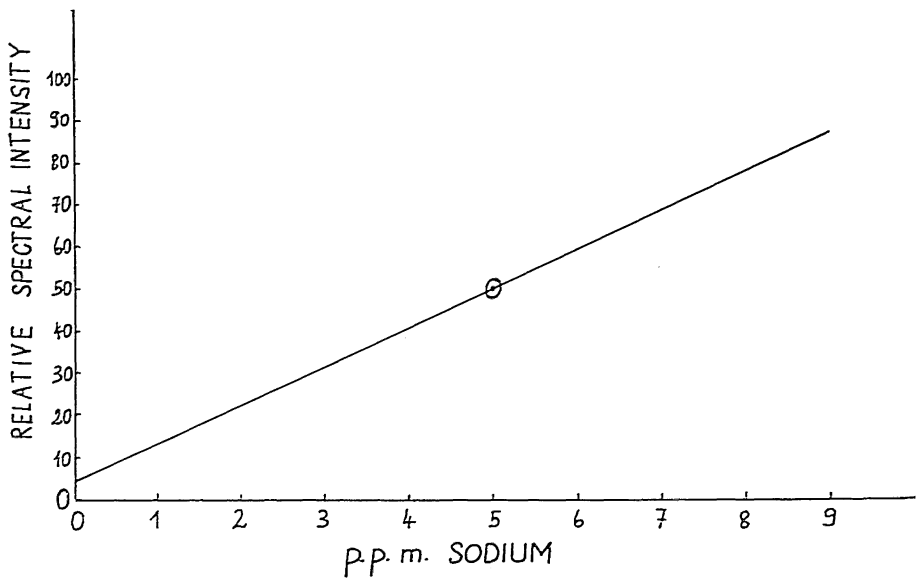


FIG. 3. Calibration curve for sodium. The curve is obtained by measuring the sodium emission (Table 1) of ten concentrations of Na ranging from 1 to 10 p.p.m., added to a standard solution containing 14 p.p.m. K, 8 p.p.m. Ca, 1.2 p.p.m. Mg, and 14 p.p.m. NH_4OH in 0.5 *N* HCl.

⊙ = reference point arbitrarily chosen to check the photometer before running an analysis.

HCl is the most suitable dilution, yielding concentrations of from 5 to 50 p.p.m. (Figure 4). For Mg analysis, a dilution of 1 : 5 with water is made up, yielding concentrations of from 10 to 60 p.p.m. of Mg in a 0.7 *N* hydrochloric acid solution (Figure 5). Before making up the dilution for Mg, the three cations Na, K, and Ca are analyzed. Then, with these known concentrations, a dilution of the sample for Mg analysis is calculated, to get close to the concentration ranges of cations of the standard solution chosen for the Mg calibration curve. It is most important that the total concentration of Na plus K plus Ca correspond as closely as possible to the concentration of 510 p.p.m. of these three cations, as used to establish the calibration curve for Mg (Figure 5). Ammonia has practically no influence in a wide concentration range.

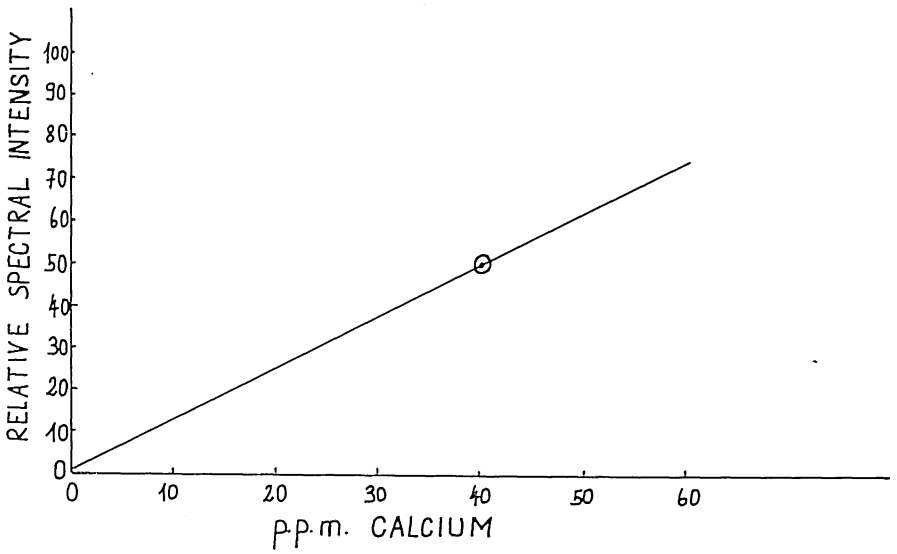


FIG. 4. Calibration curve for calcium. The curve is obtained by measuring the calcium emission (Table 1) of 12 concentrations of Ca ranging from 5 to 60 p.p.m., added to a standard solution containing 56 p.p.m. K, 14 p.p.m. Na, 4.8 p.p.m. Mg, and 56 p.p.m. NH_4OH in 0.5 *N* HCl.

⊙ = reference point arbitrarily chosen to check the photometer before running an analysis.

The diluted pure chloride solutions are analyzed with the Beckman DU spectrophotometer under conditions as pointed out (Table 1). Results of the analyses, read on the transmission dial, are compared to a calibration curve.

Calibration curves (see Figures 2, 3, 4, and 5). It is important to establish the calibration curves carefully. Every constituent in the sample to be analyzed should be present in the standard solutions used for the calibration, because every substance present interferes with the specific emission of a certain concentration of an element in the flame. For the same reason, no other substance is allowed to be present in the standard solution. Therefore, all standard solutions contain HCl, K, Na, Ca, Mg, and ammonia, in concentrations which are expected to occur in the sample to be analyzed.

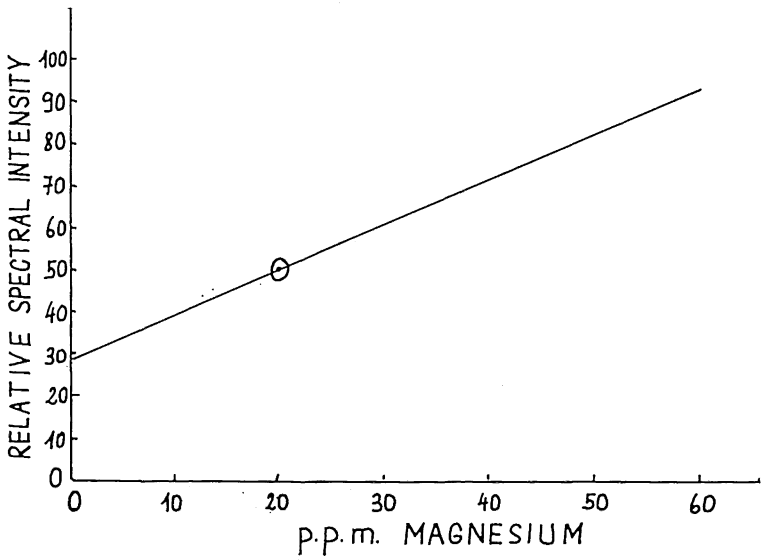


FIG. 5. Calibration curve for magnesium. The curve is obtained by measuring the magnesium emission (Table 1) of 12 concentrations of Mg ranging from 5 to 60 p.p.m., added to a standard solution containing 280 p.p.m. K, 70 p.p.m. Na, 160 p.p.m. Ca, and 280 p.p.m. NH_4OH in 0.7 *N* HCl.

⊙ = reference point arbitrarily chosen to check the photometer before running an analysis.

The calibration curves shown (Figures 2, 3, 4, and 5) are typical for analyses of milk, and they can be used only for those cations and in solutions of this standard composition.

Once the calibration curves are established, one standard solution is chosen to balance the DU spectrophotometer each time before the unknown samples are analyzed. With this standard solution, the transmission dial is set to the reference point on the calibration curve and the photometer balanced; a slight adaptation of the slit width may be necessary for accurate reproduction of standard conditions.

RESULTS AND DISCUSSION

Reproducibility of the flame photometric analysis and recovery of cations after adsorption on the Dowex 50 ion exchange resin. The trials on reproducibility and recovery experiments are shown (Figure 1 and Table 2). In Figure 1 it can be seen that known amounts of cations as chlorides, dissolved in a 100-ml. sample, had been adsorbed on Dowex 50 (see also Table 2, Experiment 1). During the elution with 3.5 *N* HCl, four fractions of 20, 10, 10, and 10 ml. had been taken and each of them analyzed for Na, Mg, K, and Ca. Under these conditions, 20 ml. of HCl are sufficient for a 100% recovery of Mg and Na; the recovery of K was complete after elution of 30 ml. of HCl, and Ca needed 50 ml. for total recovery.

Table 2, Experiment 2, shows that a salt mixture added to skimmilk is recovered quantitatively after going through the complete analytical procedure; in Experiment 3, the maximum deviations of Na, K, and Ca on the mean values

TABLE 2

Reproducibility and recovery experiments of the flame photometric analysis of cations

Experiment No.	Sample analyzed		Cations adsorbed on Dowex 50 calculated	Cations eluted from Dowex 50 found	Recovery	Maximum deviation
			(mg.)		(%)	
1	Salt mixture	K	70.0	71.0	101	+ 1
		Na	16.0	15.6	97	− 3
		Ca	40.0	40.8	102	+ 2
		Mg	6.0	5.9	98	− 2
2	40 ml. skimmilk[a]	K	(?)	65.0		
		Na	(?)	14.0		
		Ca	(?)	50.0		
		Mg	(?)	4.2		
	20 ml. skimmilk with added salt mixture[a]	K	64.5	64.0	99	− 1
		Na	14.0	13.5	96	− 4
		Ca	55.0	55.0	100	0
		Mg	4.1	3.8	93	− 7
3	Means of five different samples of 100 ml. of skimmilk	K	(?)	168		± 5.8
		Na	(?)	35		± 5.5
		Ca	(?)	119		± 7.5
		Mg	(?)	11		±12.7
4	10 g. whole milk powder *(c)*, means of three complete analyses[b]	K	(?)	122.0		± 0
		Na	(?)	24.7		± 3
		Ca	(?)	95.3		± 1
		Mg	(?)	8.8		± 2
	10 g. whole milk powder *(c)*, ashed before adsorption on Dowex 50[b]	K	122.0	122.0		0
		Na	24.7	22.0		−11
		Ca	95.3	94.0		− 1.4
		Mg	8.8	8.4		− 0.5

[a] Identical samples of skimmilk.
[b] Identical samples of whole milk powder.
[c] Nestlé whole dry milk powder (Nido).

of skimmilk analysis are larger than the error on the recovery experiments. This means that the method is suitable for detecting variations in the cation content of milk. Experiment 4 shows an excellent reproducibility of the two different preparations of the milk sample, after casein precipitation with acid as described by Jenness *(2)* and Murthy and Whitney *(4)*, and after ashing of the milk powder. Ashing of milk at temperatures above 400° C. often produces losses of Na and Cl, as observed in our Laboratory (unpublished data). This may explain the lower figure found for Na in milk ash determination.

Interference of cations in the flame spectrophotometric analysis. All constituents of a solution to be analyzed by flame spectrophotometry interfere more or less with the determination of cations. For this reason, the calibration curves should contain all constituents of the sample to be analyzed and in the same proportions. Therefore, it is important that the sample to be analyzed be purified from all undesired ingredients, as far as is possible; care should be taken, especially, not to introduce new ones. The cation adsorption on Dowex 50 with

subsequent elution with HCl seems to be an especially important step, because of the complete elimination of organic substances and the complete elimination of all anions. An accurate Ca and Mg analysis is impossible if the phosphoric acid is not eliminated quantitatively.

By this process of cation adsorption on Dowex 50, the reciprocal interferences of the cations themselves need to be considered, as well as the minor influences of HCl and NH_4OH. The concentrations of HCl and NH_4OH are held constant; therefore, they do not interfere. The ammonia, especially, has little influence.

The interference of cations is of two kinds: *(a)* Depression of emission by the presence of high concentration of other cations: *(b)* increase of the emission by other cations.

The depression of emission does not appear under the conditions described in this paper; the total concentration of cations does not exceed 1,000 p.p.m. (see Figure 5, calibration curve for Mg).

The increase of emission of one cation by other cations always is to be expected. For the Na, K, and Ca determination, those interferences would be of minor importance, because of the low total concentration of the cations and the high emission activities of these ions. For Mg, the interferences of Na, K, and Ca are important, because of the weak emission intensity of Mg and the relatively small proportion of Mg present in milk minerals, the total concentration of Ca, K, and Na being about 30 times as high as that of Mg. For accurate Mg analysis, therefore, it is necessary to analyze Na, K, and Ca first, then to calculate the concentration range of the sample and dilute to a concentration of Na plus K plus Ca, so as to obtain a concentration range as close as possible to the concentration used to establish the calibration curve. It has to be considered that the HCl content of the final dilution must be 0.7 *N* (see Figure 5).

ACKNOWLEDGMENT

The author gratefully acknowledges the help of Dr. E. Bovet of the Federal Experimental Station of Agriculture at Lausanne, Switzerland, while starting work on flame spectrophotometric determination of cations.

The analytical work was done by F. Moser, Laboratory Assistant, who contributed much.

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COMPARISON OF STANDARD METHODS OF MEASURING HOLDING TIME IN H.T.S.T. PASTEURIZERS

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SUMMARY

Three tests for the determination of holding time were compared; namely, the nickel chloride–dimethylglyoxime chemical method, the sodium nitrite–Griess-Ilosvay chemical method, and the salt conductivity method. Tests were carried out, under field conditions, on five commercial plate-type, high-temperature, short-time pasteurizers, the capacities of which ranged from 3,935 to 16,525 lb. per hour. The size of holding tubes on these units was from 1½- to 2½-in. nominal size sanitary piping, whereas one of the units had a plate-type holder section.

The methods were compared under conditions as nearly identical as circumstances would permit. Results of tests, carried out in accordance with these methods, were compared on the basis of the same flow rate, with the same type of injection being used. The point of injection of the solution used as a tracer was the same in all tests in relation to the flow of liquid entering the holding section.

The results indicate that the sodium nitrite test was more sensitive as a method of determining holding time than either the nickel chloride test or the salt conductivity test. This was more apparent in high-capacity units, using larger-sized holding tubes, and in lower-capacity units with a low holding-tube efficiency. The nickel chloride test and the salt conductivity test were shown to be of comparable sensitivity on low- and medium-capacity units, but on higher-capacity units the nickel chloride test appeared more sensitive than the salt conductivity test.

The universal adoption of H.T.S.T. pasteurization of milk, with a holding time of a matter of a few seconds, necessitates a precise and accurate method of measuring the time of holding of the milk under this system. Various methods have been used or suggested for use in the determination of holding time in H.T.S.T. pasteurizers, each having its relative advantages and disadvantages. The object of any method is to determine the minimum time in which a bacterial cell will travel through the holding tube. Recent years have seen the development of more accurate methods. This study is a comparison of two methods of determining holding time, one of which is considered to be the most accurate method available, and the other, one of the most widely used methods. The former method may be called a chemical method as it involves the injection of a chemical substance at the entrance to the holding tube which is detected at the outlet of the holding tube by a reagent which gives a colour reaction. The latter method is the electrolytic or salt conductivity method which has been standardized in the United States for the measurement of holding time in H.T.S.T. pasteurizers.

REVIEW OF THE LITERATURE

The fact that certain chemicals can be detected by a simple colour reaction forms the basis of a test for holding time devised by Dummett and Mongar *(3)*. This method depends on the injection of a saturated solution of nickel chloride and employs the organic reagent dimethylglyoxime as a detector, which gives

Received for publication January 9, 1958.

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a yellow colour or pink precipitate according to the concentration. In carrying out the test, 5 cc. of the saturated solution of nickel chloride are injected at the inlet to the holding tube by a spring-loaded syringe. A sampling valve is fitted at the outlet of the holding tube. Samples are taken at 1-sec. intervals into the sampling tubes containing 1 cc. of dimethylglyoxime solution. The presence of nickel chloride in any filled tube will be shown by the colour reaction with the reagent. The holding time in seconds thus is automatically recorded by the number of clear tubes, plus the time in seconds from injection to start of sampling. The sensitivity of this method is claimed to correspond to a dilution of 1 part of nickel chloride in 100,000 parts of water.

A variation of the Dummett and Mongar test was suggested by Botham *(1)*, who used sodium nitrite as the injected solution and the Griess-Ilosvay reagent as the detector. Sodium nitrite was chosen by Botham as a chemical which could be used to test the holding time on milk. This test was shown in laboratory experiments, with milk warmed to pasteurizing temperature, to give a recognizable pink colour in the presence of as little as one part of added sodium nitrite in two million parts of milk. In a series of tests run on the latest design of holding tube, using water and milk successively, it was shown that, whereas the nickel chloride test gave 17 sec., the sodium nitrite test gave a holding time of 16½ sec. Botham claims that the holding time for a particular piece of apparatus in turbulent flow conditions is given with sufficient accuracy both by nickel chloride and dimethylglyoxime on water, or by sodium nitrite and the Griess-Ilosvay reagent on milk. Burton and Thiel *(2)* describe the nitrite test as the most sensitive test yet available for determining holding time in H.T.S.T. pasteurizers.

Electrolytic methods of determining holding time are adapted from methods of measuring water-flow in pipes. The principles of the electrolytic flow measurement in application to H.T.S.T. pasteurizing units are given by Roger *(7)*. A saturated solution of sodium chloride was injected at the inlet to the holding tube where a pair of electrodes, joined in circuit with dry cells, and a 25 Ma. meter were situated. Another pair of electrodes connected to a similar circuit, in which a 5-Ma. meter was used, was situated at the outlet of the holding tube. In operation, one person injects the salt solution and another starts the stop watch when the needle of the ammeter in the circuit at the inlet deflects owing to the increased conductivity caused by the salt charge passing the electrode; then stops the watch when the ammeter needle at the outlet shows a deflection. A detailed description of the electrolytic method is given by Fay and Fraser *(4)*, who compared the manual system of timing with timing by means of an automatic electric timer. Over a series of 11 readings, they found a maximum variation of 1 sec. with manual timing, and 0.6 sec. with automatic timing. Weber *(9)* observed that the salt test is a practical means of determining the minimum holding time when operating with water. It has the disadvantage that it is quite complicated, and can not be used directly on milk or milk products. Robinson and Moss *(6)* note that the detection of a salt charge in the holding tube was somewhat slower by an automatic timer than by a milliammeter. The difference

in time between the two instruments varied from 0.9 to 3.50 sec., and the difference increased with decreasing velocity of flow. A study of possible variations which might be introduced into the salt test was made by Jordan *(5)*. He found that for an average velocity of water at 160° F. of 1.268 ft. per second in 2½-in. sanitary pipe, all injected volumes of saturated sodium chloride solution from 15 to 150 cc. gave holding times which agreed within about 0.1 sec. A decrease in the injected volume below 15 cc. apparently resulted in increasing the holding time. Jordan also observed that the direction in which the salt solution was introduced into the tube affected the apparent holding time. A difference in apparent holding time of about 0.7 sec. was found between injections with, and injections against, the stream. A spray-type nozzle and a straight tube injection across the flow were found to give comparable results. When compared under identical conditions, manual (or stop watch) timing and automatic timing were found to give holding times which agreed closely. From the results of a series of experiments showing that the organism, *Escherichia coli*, and the sodium chloride are transported at the same rate by water at 80° F., Jordan concludes that, if satisfactorily sensitive means are used for detection, salt can be used for the estimation of the holding time of bacteria.

The necessity of having a standardized test for holding time was recognized in the United States in 1950, when the 3A Standard method for determining the holding time in H.T.S.T. pasteurizers by the salt conductivity test was formulated. This standard gives approved apparatus and materials to be used in the test and outlines the method to be followed, step by step. Formulae are also given for computing the holding time for milk from that obtained when the unit is operated on water.

EXPERIMENTAL PROCEDURE

This comparative study was carried out on selected plate-type pasteurizing units. The holding time was determined on each unit tested by means of the nickel chloride–dimethylglyoxime test of Dummet and Mongar *(3)*, the sodium nitrite–Griess-Ilosvay test of Botham *(1)*, and the 3A Standard salt conductivity test *(8)*.

The equipment used in the chemical tests, that is, the nickel chloride and sodium nitrite tests, was manufactured as a standard test-kit by the A.P.V Company Ltd., Sussex, England. Minor alterations were made on the injection and sampling devices supplied, to ensure that the point of injection or sampling came to the center of the flow stream through any size of sanitary connections to which the devices were fitted. Spray-type injection nozzles were used in all tests, because this method of injection is considered most accurate under varying circumstances and is also the method adopted in the 3A Standard salt conductivity test. Holding time was determined from the results of six chemical tests on each unit tested. In three of these tests, sampling was begun 10 sec. after injection of the tracer and in the remaining three, 10½ sec. after injection. This allowed the holding time to be expressed to the nearest 0.5 sec. After each test the unit was run for approximately 5 min., to ensure that the injected solution was cleared from the system.

Preliminary tests were carried out to determine the sensitivity of the reagents used in the chemical tests. These confirmed the claims made in respect to the tests; namely, that the Griess-Ilosvay reagent is capable of detecting as little as one part of sodium nitrite in 10 million parts of water, and that dimethylglyoxime is capable of detecting as little as one part of nickel chloride in 100,000 parts of water, under practical operating conditions.

The equipment and method used in the salt conductivity test conformed to the standards specified in the 3A Standards for this test. The electrodes and automatic Solu-Bridge Flow Timer employed in the tests were manufactured by Industrial Instruments Inc., New Jersey, U.S.A. The automatic Solu-Bridge Flow Timer responded to a concentration as low as 4 p.p.m. of salt in water. A response to at least 10 p.p.m. of salt in water is specified for the conductivity instrument used in the salt test by the 3A Standard method. The holding time on each unit tested by the salt conductivity test was taken as the average of ten successive tests, with a variation between the minimum and maximum values not exceeding 0.5 sec.

Holding-time determinations, by the methods outlined, were made on commercial plate-type, high-temperature, short-time pasteurizers, selected to give a wide range of capacities and size of holding tube. Five units were included in the study, having capacities of 3,935 to 16,525 lb. per hour. The units had tubular holding sections with one exception; this unit had a box plate-type holding section incorporated in the press.

During test runs, the units were operated on water at approximately milk pasteurization temperature, with a constant head of water maintained in the pump feed-tank during operation. The holding time on each unit was first determined by the chemical method, the unit closed down and the injection nozzle and sampling valve replaced by the salt conductivity test electrodes. On restarting, the holding time was determined by the salt conductivity test. Holding-time determinations were done only after the unit had attained equilibrium temperature and flow conditions.

The flow rate of the unit was checked regularly during the period of the test run. The discharge from the sampling valve during the chemical test was taken into account in computing the flow rate of the unit during that test. However, a discrepancy was noted in the flow rates of the units computed during the periods when the two tests were being carried out, even though the units were being operated under identical conditions. It was concluded, therefore, that the discrepancy, an increase in the flow rate during tests by the chemical method, was caused by the release of pressure within the system, due to the discharge through the sampling valve used in the chemical method. This had the effect of reducing the discharge head of the unit, causing the discharge of the pump to increase. This effect was greatest when a Waukesha positive displacement pump was used; whereas, the discharge was much more constant when a homogenizer was used.

In order that the holding times obtained by the different methods could be compared on the basis of the same flow rate, it was decided to adjust the holding

times given by the salt test, to the higher flow rate measured under conditions of testing by the chemical test. The validity of carrying out this adjustment was established by a series of tests carried out on a plate-type holding section. These tests showed that the holding time given by the salt conductivity test could be adjusted by direct calculation to the higher flow rate measured during the chemical test, within a limited range of about 100 lb. per hour. This was the order of the discrepancy in flow rate taken during the two tests. Thus, the holding time given by the salt conductivity test on the units included in this study has been adjusted in this manner to give a test comparable with the holding time given by the chemical method. In the presentation of results, this adjusted salt conductivity test has been given to the nearest lower 0.5 sec. This was done to give a truer comparison of the tests, because the chemical test results also are expressed in this way due to the method of sampling.

The efficiency of the holding tube of each unit tested was calculated, using the following formulae:

$$\bar{v} = \frac{w}{\rho\, a\, t} \tag{1}$$

where $\bar{v}$ = average velocity, ft/sec
w = weight of water collected, lb.
ρ = density of water at measured temperature, lb/ft^3
a = area of pipe, ft.^{2}
t = time for collecting water, sec.

$$v_{H.T.} = \frac{L}{H.T.} \tag{2}$$

where $v_{H.T.}$ = velocity of fastest measured particle, ft/sec
L = length of tube between injection and detection point, ft.
H.T. = measured holding time, sec.

$$\text{Holding-tube efficiency} = \frac{v}{v_{H.T.}} \tag{3}$$

All holding-tube efficiencies were calculated on the basis of the holding time measured by the Griess-Ilosvay test and the flow rate measured during that test. When obtaining the length of holding tubes, the equivalent length of straight piping for 90°-sweep ells in the tube was taken from measurements given by the New York State Department of Health.

RESULTS AND DISCUSSION

The following results are given for each individual pasteurizing unit, together with any relevant details of the unit:

Unit No. 1. The holding tube of this unit was 30.82 ft. long, in 1½-in. sanitary pipe with C.I.P. fittings. The calculated efficiency of the tube was 87%. The milk pump was a Creamery Package homogenizer, driven by a 10-h.p. electric motor, having a maximum operating pressure of 2,000 p.s.i.g. During tests, the homogenizer was operated with no pressure on the homogenizing valve. The unit was operated on water at 163° F. ± 0.5° at a flow rate of 3,935 lb. per hour.

The results of the holding time tests are given (Table 1). They indicate that, under these circumstances, the three tests have similar sensitivities.

TABLE 1
Summary of holding time tests

Unit	Flow rate	Holding tube efficiency	Holding time		
			Sodium nitrite	Nickel chloride	Salt conductivity
	(lb/hr)	*(%)*	*(sec.)*		
1	3,935	86	16	16	16
2	4,274	75	18	18.5	18.5
3	8,001	77	16	17	16.5
4	13,153	86	14	14.5	14.5
5	16,525	83	17.5	18	18.5

Unit No. 2. The holding tube of this unit was 15.10 ft. long, of three sizes of sanitary piping; namely 2, 2½, and 3 in. The calculated efficiency of the tube was 74.6%. The milk pump was a Waukesha positive displacement pump, with Reeves variable speed drive, driven by a 1.5-h.p. electric motor. The unit was operated on water at 165° F. ± 0.5° at a flow rate of 4,274 lb. per hour. The results are given (Table 1).

Under these conditions, the results indicate a higher sensitivity of the sodium nitrite test and a lower, but comparable, sensitivity of the nickel chloride and salt conductivity tests.

Unit No. 3. A plate-type pasteurizing unit in which the holding section was made up of eight, ¾-in. holder plates, which consisted of a central frame enclosed by two plates of design similar to those used in other sections of the unit. The calculated efficiency of the holder section was 77.2%. The milk pump was a Waukesha positive displacement pump with Reeves variable speed drive, driven by a 1.5-h.p. electric motor. The unit was operated on water at 163° F. ± 0.5°, at a flow rate of 8,001 lb. per hour. The results of holding-time tests on this unit are given (Table 1).

These results show the sodium nitrite test to have a higher sensitivity than the nickel chloride test and the salt conductivity test. The salt conductivity test is shown to give a holding time of 0.5 sec. less than the nickel chloride test. It is pointed out in this case, however, that the salt conductivity test after adjustment to flow rate was 16.83 sec. Under the system selected for presenting results, this became 16.5 sec. Thus, it is probable that the holding times measured by the salt conductivity test and the nickel chloride test were similar under these conditions.

Unit No. 4. The holding tube of this unit was 51.09 ft. long, in 2-in. sanitary piping with C.I.P. fittings. The calculated efficiency of the tube was 86%. The milk pump was a Cherry-Burrell homogenizer, driven by a 50-h.p. electric motor, with a maximum operating pressure of 2,500 p.s.i.g. During tests, the homogenizer was operated on water at 163° F. ± 0.5° at a flow rate of 13,153 lb. per hour, and with no pressure on the valve. Results are given in Table 1.

These results indicate that the sodium nitrite is more sensitive than the nickel chloride and salt conductivity tests, under conditions of high holding-tube efficiency, with larger flow rates. The controlling factors in this case ap-

peared to be the greater dilution of the injected tracer in the holding tube, and the greater sensitivity of the sodium nitrite–Griess-Ilosvay reaction, enabling earlier detection of the foremost particles of tracer passing through the tube.

Unit No. 5. The holding tube of this unit was 51.56 ft. long, in 2½-in. sanitary piping. The calculated efficiency of the tube was 83.3%. The milk pump was a Waukesha positive displacement pump, with Reeves variable speed drive, driven by a 2-h.p. electric motor. The unit was operated on water at 163.5° F. ± 0.5°, at a flow rate of 16,525 lb. per hour. Results are given (Table 1).

These results bear out the results found with Unit No. 4. In this case, with the larger size, 2½-in. holding tube, and a high holding-tube efficiency, the sodium nitrite test again is shown to exhibit a higher sensitivity of holding-time determination than either the nickel chloride test or the salt conductivity test. In addition, under these conditions, the nickel chloride test is shown to be more sensitive than the salt conductivity test.

CONCLUSIONS

The relative sensitivity of the tests studied is a function of the size of the unit and the size and the efficiency of the holding tube.

Of the chemical tests used, the sodium nitrite test was preferred. The red precipitate in the nickel chloride–dimethylglyoxime reaction stained the sampling tubes and rack which, in subsequent tests, might give a false impression of colour. This was not so for the sodium nitrite–Griess-Ilosvay test.

The salt conductivity test was more easily adapted to varying conditions than the chemical test and it was not more difficult to operate. The reagents for the chemical test required greater care and time in preparation and handling than the salt solution for the salt conductivity test; the salt conductivity test could be done in much less time than the chemical test.

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A TEST FOR THE DETECTION OF LACTIC BACTERIOPHAGE USING 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE [1]

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SUMMARY

In this study, 2,3,5-triphenyltetrazolium chloride (TTC) was used to measure inhibition of lactic cultures by bacteriophage. The procedure follows: Place 8.7 ml. of 10% sterile reconstituted nonfat dry milk (NDM) in each of two sterile test tubes. To each tube add 0.2 ml. of a lactic culture diluted 1:1 with sterile 10% NDM. Add 1 ml. of serially diluted phage filtrate to one tube. To the other tube, remaining as the control, add 1 ml. of sterile distilled water. Place sterile rubber stoppers in the tubes and invert twice to mix the contents. Incubate the tests for 4 hr. at 32° C. in a water bath. Add 0.1 ml. of 1:25 aqueous solution of TTC per tube. Invert the tubes twice and incubate again for 30 min. at 32° C. Compare the color in the tube with added filtrate to the control tube. If less color is developed than in the control, inhibition has occurred. Results of this test are available in 4.5 hr. Its sensitivity for bacteriophage is comparable, and in some cases better, than a litmus milk test *(8)* requiring from 12 to 16 hr. With mixed-strain lactic starter cultures, the TTC test indicates some cases of phage inhibition in 4.5 hr. that are not evident after extended incubation in the litmus milk test. By using unheated filtrate and filtrate heated to 80° C. for 10 min., this test can be used to distinguish between inhibition of lactic cultures caused by heat-stable inhibitors and bacteriophage.

The economic losses caused by bacteriophage in the production of cultured dairy products are well-known. Reviews by Babel *(2)*, Elliker *(3)*, and Whitehead *(9)* cover the subject extensively.

Two techniques have been used to isolate or detect bacteriophage: counting plaques on solid or semisolid media inoculated with serially diluted phage filtrates and susceptible cells; and changes in acidity or oxidation-reduction potential in broths or milk media containing the test organisms and phage filtrates.

The TTC test, an effective indicator of bacterial growth *(7)*, was used in this study to detect bacteriophage action on lactic organisms. It was believed that the TTC test might be applicable for checking sensitivity of single lactic strains to phage strains, and for testing for phage as a cause of starter failure in making cultured dairy products. A litmus milk method has been reported as a reliable method of estimating phage concentrations *(8)*. This method was used as a standard of comparison for adaptation of the TTC test for phage detection.

METHODS

The cultures used in this study were obtained from two sources: the single strain *Streptococcus lactis* cultures from E. B. Collins, University of California, Davis, and the mixed-strain lactic cultures from Chris Hansen's Laboratory, Milwaukee, Wisconsin.

Received for publication December 13, 1957.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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All cultures were propagated in a 10% reconstituted nonfat dry milk medium sterilized at 121° C. for 15 min. in the autoclave. The cultures were transferred daily to retain optimum activity. *S. lactis* single strains were incubated for from 8 to 10 hr. at 32° C. The mixed lactic cultures were used for inoculum after incubation for from 14 to 16 hr. at 22° C.

Bacteriophage strains specific for each of the *S. lactis* single strains also were obtained from E. B. Collins, University of California. Reserve stocks of phage filtrates were prepared, using #03 Selas candles for filtering, to obtain sterility. The phage filtrates were stored at approximately 4° C. They were checked for sterility by adding 1 ml. of the filtrate to 10 ml. of litmus milk and then incubating these samples at 32° C. for 48 hr. After the 48 hr., the litmus milk with added phage filtrate was compared to the control with no phage filtrate added, to measure sterility.

Titers of the phage filtrates were determined by using the litmus milk method proposed by Nelson *(8)*. The determinations were carried out in triplicate at each dilution. The greatest dilution of the phage filtrate with sterile distilled water causing inhibition of growth in at least two of the triplicate determinations was designated the titer. Dilutions of the original phage filtrates up to 10^{-11} were tested.

A TTC solution containing 4 g. of TTC in 96 ml. of sterile distilled water was made; it was stored at 4° C. when not in use. When 0.1 ml. of this solution was added to 10 ml. of medium, a final concentration of approximately 0.4 mg/ml of TTC was established.

A permanent color standard was made by mixing equal parts of aqueous solutions of 0.1% methyl orange and 0.25% safranin and adding the mixture at three levels to 10 ml. of 10% reconstituted sterilized NDM. The three levels of color standard used are shown (Table 1) with the color ratings associated

TABLE 1

Arbitrary color standard for TTC test using 0.1% methyl orange and 0.25% safranin

Milk	Dye mixture	Color rating	Corresponding chip in Munsell Book
(ml.)			
10.0	0.015	(+)	(10.R) 8/4
10.0	0.060	(++)	(5.OR) 7/6
10.0	0.180	(+++)	(5.OR) 6/10

with almost complete inhibition (+), slight inhibition (++), and no inhibition of bacterial growth (+++) during the TTC test. Proper color chips from the Munsell Book of Colors *(5)* were selected and mounted on a card for rapid reference.

The following factors were varied in the TTC test, to find conditions associated with minimum time for test performance, and with sensitivity comparable to the litmus milk method: *(1)* Amount of inoculum; *(2)* concentration of TTC; *(3)* time of adding TTC, and *(4)* total length of time for the test.

After completing preliminary experiments to establish test conditions, the following procedure for the TTC test was used to compare it further with the litmus milk method.

Recommended TTC Method

1. Place 8.7 ml. of 10% sterile reconstituted NDM in each test tube.
2. Bring the tubes' contents up to 32° C. in the water bath.
3. To each tube add 0.2 ml. of a culture diluted 1:1 with sterile 10% NDM.
4. Add 1 ml. of diluted phage filtrate to one tube and 1 ml. of sterile distilled water to the control tube.
5. Place sterile rubber stoppers in tubes and invert twice to mix the contents.
6. Incubate the tubes for 4 hr. at 32° C.
7. Add 0.1 ml. of a 1:25 solution of TTC.
8. Invert twice and incubate for an additional 30 min.
9. Compare the tubes to a control tube with no phage filtrate and rate them according to the color standard.
10. Any tube showing less color development than the control indicates inhibition of bacterial growth.

RESULTS AND DISCUSSION

Factors investigated in determining optimum conditions for the test:

1. *Per cent inoculum.* Since sufficient inoculum is needed for visible color development in a reasonable time, levels of 1, 2, and 4% were used in developing the TTC test. Using a 1% level of inoculum, phage could be detected in higher dilutions of phage filtrates than with 2 or 4% inoculum. With 1% inoculum, adequate growth took place after 4 hr. of incubation at 32° C., to give sufficient color development when TTC was added. Higher levels of inoculum usually coagulated the milk in the control tubes in less than 4 hr. This makes the test ineffective, because added TTC is not reduced; therefore, color does not develop when coagulation has occurred.

2. *Concentration of TTC.* The intensity of color development is in proportion to the numbers of actively metabolizing bacterial cells and the amount of TTC available for reduction. Laxminarayana *(4)* has shown this with several lactic cultures. Neal *(7)* reported that levels of TTC above 1.2 mg/ml caused reduction in color development by an *S. lactis* culture. Mustakallio *(6)* also stated that levels of TTC above 1.0 mg/ml caused inhibition of lactic cultures.

Since the TTC solution is added after a 4-hr. delay and a control is used and the test is read in 30 min., the problem of inhibition is not important. Neal *(7)* used a *Streptococcus thermophilus* culture which is inhibited by levels of TTC lower than those which inhibit a *S. lactis* culture.

The higher the concentration of TTC present, the more the reduction is affected by light *(4)*. Therefore, the lowest amount of TTC giving a good in-

tensity of color should be the most desirable. In this study, levels of TTC of 0.4, 0.8, and 1.2 mg/ml final concentration in the medium were tried.

The color intensity developed was greater using the higher levels of TTC than using 0.4 mg/ml. A control for each level of TTC was always used as a comparison to cancel this error. In some instances, phage was detected at higher filtrate dilutions when using 0.4 mg/ml of TTC than when using 0.8 mg/ml. It appeared that 0.4 mg/ml was a sufficiently high level of TTC to get excellent test results. By keeping TTC concentrations low, light sensitivity was reduced and problems of inhibitions of bacterial growth by the added TTC were lessened.

3. *Time of TTC addition.* Neal *(7)*, in his use of the TTC test for antibiotics with *S. thermophilus* as test culture, found that a delay of 2 hr. before TTC was added to the growing culture gave greatest sensitivity. This same principle was true in the use of the test for bacteriophage detection.

In these experiments, TTC was added at three intervals—at the beginning of incubation, after 2 hr., and after 4 hr. All tests were read at the end of 4.5 hr. incubation. When TTC is added at the beginning of incubation, apparently the organisms cause reduction of the TTC and color development before being attached and lysed by the phage. As a result of this, phage was detected only at dilutions of filtrate up to 10^{-5} when the TTC was added at the start of the incubation period. If a delay of 2 hr. after the initial incubation was allowed before the TTC was added, phage was detected in filtrate dilutions up to 10^{-8}. If a delay of 4 hr. was allowed, phage was detected in dilutions up to 10^{-10}.

This trend was even more evident in the case of one *S. lactis* culture. This culture had a high activity and yielded a phage filtrate with a titer of 8. It had a tendency to overgrow the phage and, when the TTC was added at 0 and 2 hr., phage could be detected only in filtrate dilutions up to 10^{-3} and 10^{-4}. When the TTC was added after 4 hr., the phage had developed and was detected up to dilutions in filtrate of 10^{-7}.

4. *Length of time for the test.* Bacteriophage undergoes a latent period, or lag phase, in its development similar to the lag phase in the growth of bacterial cells. This period of adsorption before development and lysis is an important factor to be considered in tests for phage action. Zehren and Whitehead *(10)* reported 40- to 60-min. latent periods for the six phage strains they tested. Zehren and Whitehead *(10)* also have shown the importance of the burst size, or the number of phage particles, released when a bacterial cell undergoes lysis, on the increase in phage concentrations. Sufficient time must be allowed for the phage to increase in numbers, especially when phage filtrates are diluted out and the concentration of phage particles is low.

From results obtained in this study, it appears that an interval of about 4 hr. of incubation at 32° C. was necessary for the phage development period. For example, at 2.5 hr. time, phage was usually detected only in dilutions of phage filtrate up to 10^{-7}, but at 4.5 hr. the phage in the higher dilutions had increased in numbers and was detected at dilutions up to 10^{-10}, using a 1% inoculum. Increasing the length of time to 6.5 hr. for the test did not increase the sensi-

tivity to any extent. This same trend was true with all cultures used, both single-strain *S. lactis* and mixed-strain lactic cultures.

COMPARISON OF THE TTC AND LITMUS MILK METHODS FOR DETERMINING TITERS OF PHAGE FILTRATES

The litmus milk method has been used by several investigators *(1, 8, 9)* to estimate titers and numbers of particles in serially diluted phage filtrates. An extremely small inoculum, one drop of culture diluted 1:8 in sterile milk medium, was used per 10 ml. of litmus milk and an incubation period of from 12 to 16 hr. was used.

In this study, an attempt was made to obtain comparable results with the TTC method, using a total time of 4.5 hr. for the test. The titers obtained, using four single *S. lactis* strains and two mixed lactic cultures and their respective phages, are shown (Table 2). Results with three of the four *S. lactis* single Strains 4, 5, 7 were identical for both methods. Phage was detectable in dilutions of the various filtrates to 10^{-10}, or a titer of 10 was found. For Strain No. 6, the titer by the TTC method was one dilution less than that by the litmus milk method. This may have been a result of the phage having a longer latent period and the culture having a high activity. As a result of this, it required more time for the phage to develop, cause lysis, and show dominance over the culture.

TABLE 2

Comparison of the sensitivity of the TTC method and the litmus milk method for determining titers of phage filtrates

Culture	Phage filtrate	Highest phage dilution causing inhibition	
		TTC method	Litmus milk method
4	F4	10^{-10}	10^{-10}
5	F5	10^{-10}	10^{-10}
6	F6	10^{-7}	10^{-8}
7	F7	10^{-10}	10^{-10}
3	F4	10^{-3}	No inhibition at 10^{-1}
28	F6	10^{-5}	No inhibition at 10^{-1}

Cultures 3 and 28 were mixed-strain starter cultures, which proved to be sensitive to some degree to the two phages used. Probably only one or, at the most, two of the single strains in each of the mixed strain cultures were sensitive to a phage used. This may be the primary reason why both Cultures 3 and 28 grew over the phage, even at filtrate dilution of 10^{-1}, when the litmus milk method was used. Inhibition by phage filtrate dilutions of 10^{-3} for No. 3 and 10^{-5} for No. 28 was shown when the TTC method of 4.5 hr. of incubation was used. These results are averages of five trials and are presented (Table 2).

This may be a significant point, in that at the present time a recommended procedure to detect phage in cheese plants is to place some susceptible culture and whey from suspected slow vats in sterile milk medium and incubate this at 22° C. for 24 hr. If coagulation occurs, phage is not suspected *(3)*. This may give the cheesemaker a false sense of security, because an active phage may be present which is able to lyse one or two strains in the mixed lactic starter, yet

the remaining unsusceptible strains will produce acid and coagulate the milk in 24 hr. Results (Table 2) show that a susceptible phage with titer of 10^{-5} was not detected after a 12- to 16-hr. incubation period in litmus milk. It was evident after 4.5 hr., using the TTC method, before the unsusceptible *S. lactis* strains developed and caused coagulation.

Results of testing the sensitivity, by both the TTC and litmus milk methods, of 15 single strains of *S. lactis* to two unknown phage filtrates obtained from Wisconsin cheese factories, are shown (Table 3). Usually, the results of the TTC test compared favorably with the results by the litmus milk method. Some strains did show sensitivity to a phage filtrate in the TTC test, but not in the longer litmus milk method. This may be a result either of the single strain being contaminated, or of the organism being only moderately susceptible to the phage and possessing the ability to outgrow it. The ratio between the amount of inoculum and the concentration of phage could be another factor causing some differences between the two tests.

TABLE 3

Results of TTC and litmus milk tests for the sensitivity of 15 single strains of S. lactis to two unknown phage strains obtained from whey

S. lactis strain	TTC method[a]			Litmus milk method[b]		
	Control	Phage XF added	Phage AF added	Control	Phage XF added	Phage AF added
1	3+	−	−	+	−	−
2	3+	2+	−	+	−	−
3	3+	−	1+	+	−	−
4	3+	3+	3+	+	+	+
5	3+	−	1+	+	−	−
6	3+	−	3+	+	−	+
7	3+	1+	3+	+	−	+
8	3+	−	3+	+	−	+
9	3+	−	−	+	−	−
10	3+	−	3+	+	−	+
11	3+	3+	−	+	+	−
12	3+	2+	1+	+	−	−
13	3+	2+	3+	+	+	+
14	3+	2+	1+	+	+	+
15	3+	3+	3+	+	+	+

[a] −, very strong inhibition; 1+, strong inhibition; 2+, mild inhibition, 3+, no inhibition.
[b] +, no inhibition; −, inhibition.

USE OF THE TTC TEST TO DIFFERENTIATE BETWEEN INHIBITION CAUSED BY HEAT-STABLE INHIBITORS AND BACTERIOPAHGE

The test can be a means of determining which of the two inhibitory agents are responsible for a starter failure, by making duplicate determinations. In one determination, use the suspected whey filtrate or milk as obtained; in the other, add the suspected filtrate or milk after heating it to 80° C. for 10 min. When the heated filtrate causes inhibition of the particular starter culture from above, heat-stable inhibitors which are not destroyed at 80° C. for 10 min. are probably responsible for the starter failure. The problem is almost surely bacteriophage when inhibition occurs when the unheated filtrate is used, but not when the heated filtrate or milk is added. By using this test, the cause of starter

failure could be determined and corrective measures started before milk was received on the following day.

ACKNOWLEDGMENTS

This study was supported by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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COMPARATIVE ACCURACY OF SMALL LACTOMETERS FOR DETERMINING THE TOTAL SOLIDS OF INDIVIDUAL COWS' MILK [1,2]

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SUMMARY

Milks were obtained monthly from approximately 350 cows of five dairy breeds. Averaged total solids measured with the small Watson lactometer did not differ significantly (except three Brown Swiss milks, 6% fat) from those measured gravimetrically. Correlations between the small Watson lactometer and gravimetric measurements exceeded 0.9 when fat levels were ignored, were lower when the data were grouped by fat percentages, and mostly were statistically significant. Standard errors of estimate ranged from 0.14 to 0.39, indicating errors in total solids equivalent to 0.12% fat by the Babcock method. Field use of the small Watson lactometer can be recommended, provided temperature and reading techniques are controlled.

Average total solids measured by the small Quevenne lactometer differed significantly from the gravimetric measurements in milk from several breed and fat level groups. Correlations and standard errors were of the same magnitude as those obtained with the small Watson lactometer. A significant interaction between methods (gravimetric vs. Quevenne lactometer) and fat levels was obtained when measurements for all breeds were combined, indicating an incapacity of the small Quevenne lactometer and/or the estimation equation to measure accurately total solids at various fat levels. This bias was positive at low fat and negative at high fat levels, indicating that the small Quevenne lactometer is not as accurate as the Watson lactometer.

Lactometric procedures for measuring total solids have been criticized because of inaccuracies and the relatively large quantities of milk required for their use. The recent availability of two lactometers requiring small quantities of milk suggested a study of the measurement of total solids with these lactometers compared with a standard gravimetric method. Such lactometers, if sufficiently accurate, would provide a rapid test for the total solids of individual cows' milk.

This study evaluated two small lactometers as a method for measuring the total solids of milks from individual cows of five dairy breeds.

EXPERIMENTAL METHODS

Samples. Four-ounce milk samples were collected monthly by the DHIA supervisor, from the Michigan State University dairy herds (five breeds) and seven cooperator herds (one Guernsey, five Holstein, and one Jersey herd). The distribution of cows by breed was eight Ayrshire, 30 Brown Swiss, 68 Guernsey, 180 Holstein, and 62 Jersey.

Measurements. Fat values for each sample were obtained from official DHIA records. Total solids measured by a gravimetric method on the same milk sample

Received for publication January 30, 1958.

[1] Journal Article No. 2196 from the Michigan Agricultural Experiment Station.

[2] These studies were supported in part by the Dairy Cattle Research Branch of the Agricultural Research Service, USDA.

were considered as reference values. The two lactometers used in this study to estimate total solids were: (a) the small Watson-pattern lactometer, read at 102° F., approximately 6 in. long, and requiring about 4 oz. of sample *(3)*; and *(b)* a small Quevenne lactometer of the type in official use in the Netherlands, to be read at 68° F. after previous warming to 102° F., approximately 8 in. long, and requiring about 4 oz. of sample *(2)*. Estimation of total solids from the lactometers noted above was computed as follows:

$$\text{Lactometer } (a) \quad \text{T.S.} = 1.33\,\text{F} + \frac{273\,\text{L}}{\text{L} + 1000} - 0.40 \qquad (3)$$

$$\text{Lactometer } (b) \quad \text{T.S.} = 1.23\,\text{F} + \frac{260\,(\text{L} + 1.8)}{\text{L} + 1000} \qquad (2)^{3}$$

F = Babcock fat %. L = Lactometer reading in degrees.

TABLE 1

Total solids measured by the small Watson lactometer and gravimetric methods, by breeds and fat levels (February through June, 1957)

Breed	Fat levels (%)							
	<3	3	4	5	6	7	8	All
Holstein								
No. samples	11	469	191	12	1	.....	.....	684
Av. fat	2.7	3.5	4.3	5.2	6.0	.....	.....	3.8
Av. T.S. (G)[a]	11.06	12.13	13.15	14.21	15.92	.....	.....	12.44
Av. T.S. (L)[a]	11.17	12.14	13.15	14.33	15.61	.....	.....	12.45
Guernsey								
No. samples	1	16	188	94	24	2	.....	325
Av. fat	2.4	3.7	4.4	5.3	6.2	7.4	.....	4.8
Av. T.S. (G)	10.43	12.48	13.56	14.63	15.78	16.95	.....	13.99
Av. T.S. (L)	10.36	12.52	13.48	14.61	15.86	16.66	.....	13.94
Jersey								
No. samples	.....	.....	46	123	54	10	.....	233
Av. fat	.....	.....	4.6	5.5	6.3	7.2	.....	5.6
Av. T.S. (G)	.....	.....	14.01	15.05	16.08	17.01	.....	15.17
Av. T.S. (L)	.....	.....	13.97	15.07	16.16	17.21	.....	15.20
Brown Swiss								
No. samples	1	44	51	7	3	.....	.....	106
Av. fat	2.7	3.6	4.4	5.3	6.2	.....	.....	4.2
Av. T.S. (G)	11.09	12.60	13.59	15.04	16.35	.....	.....	13.33
Av. T.S. (L)	10.88	12.52	13.72	15.05	15.60[b]	.....	.....	13.33
Ayrshire								
No. samples	.....	17	17	2	.....	.....	1	37
Av. fat	.....	3.6	4.3	5.2	.....	.....	8.5	4.2
Av. T.S. (G)	.....	12.51	13.42	14.13	.....	.....	18.65	13.18
Av. T.S. (L)	.....	12.44	13.18	13.86	.....	.....	17.69	13.00
All Breeds								
No. samples	13	546	493	238	82	12	1	1,385
Av. fat	2.7	3.6	4.4	5.4	6.3	7.3	8.5	4.4
Av. T.S. (G)	11.02	12.19	13.44	14.83	16.00	17.00	18.65	13.35
Av. T.S. (L)	11.08	12.19	13.41	14.84	16.05	17.12	17.69	13.34

[a] Average T.S. (G) and (L) refer to average total solids obtained by the gravimetric and lactometric methods, respectively.

[b] Significantly different from average total solids measured gravimetrically at P = 0.05.

[3] Equivalent to the reported estimation equations:

$$\text{D} = 1.23\,\text{V} + 2.6 \times 100\,(d^{20} - 0.9982)/d^{20}$$

where V and d^{20} refer to fat per cent and density of the milk at 20° C., respectively.

RESULTS AND DISCUSSION

Since fat levels and breed characteristics are traditionally considered as the major causes of variations in total solids, these two factors were considered in comparing the lactometric procedures with the gravimetric measurements.

Small Watson lactometer. The results of the total solids measurements using the small Watson lactometer and the gravimetric method are presented (Tables 1 and 2). The average lactometer measurements were not significantly different from the gravimetric measurements, except in three Brown Swiss samples at the 6% fat level, when fat levels and/or breeds were considered. The correlations between total solids measured lactometrically and gravimetrically were statistically significant, except for Guernsey samples at 6% and Brown Swiss samples at 5 and 6% fat levels. These correlations ranged from −1.0 to 1.0 for fat levels within breeds, from 0.90 to 0.94 for breeds when fat levels were ignored, and from 0.54 to 0.85 for fat levels when breeds were ignored. A correlation of 0.96 was obtained when breeds and fat levels were ignored. Standard errors of estimate ranged from 0.14 to 0.39 for fat levels within breeds, from 0.28 to 0.40 for breeds when fat levels were ignored, and from 0.23 to 0.39 for fat levels when breeds were ignored. A standard error of estimate of 0.36 was obtained when breeds and fat levels were ignored.

Watson *(3)*, using his large lactometer, reported 59 tests on individual cows of four dairy breeds with an average deviation of +0.05% and a standard error of estimate of 0.13. In a previous study, the authors *(1)* reported an average deviation of +0.20% and a standard error of estimate of 0.40 when a lactometer of the type employed by Watson was used on 1,454 Jersey samples. Correspond-

TABLE 2

Correlations and standard errors between total solids measured gravimetrically and with the small Watson lactometer, by breeds and fat levels (February through June, 1957)

Breed	Fat levels (%)						
	<3	3	4	5	6	7	All
Holstein							
r[a]	0.73	0.84	0.82	0.83	.....	.....	0.92
SE[b]	0.22	0.23	0.27	0.20	.....	.....	0.28
Guernsey							
r	.....	0.63	0.71	0.83	0.32[c]	1.00	0.90
SE	.....	0.25	0.31	0.24	0.26	.....	0.40
Jersey							
r	.....	.....	0.67	0.74	0.65	0.87	0.91
SE	.....	.....	0.27	0.30	0.39	0.28	0.39
Brown Swiss							
r	.....	0.84	0.71	0.41[c]	−0.96[c]	.....	0.92
SE	.....	0.26	0.33	0.30	0.14	.....	0.39
Ayrshire							
r	.....	0.92	0.66	−1.00	.....	.....	0.94
SE	.....	0.18	0.39	.....	.....	.....	0.36
All Breeds							
r	0.79	0.85	0.78	0.81	0.54	0.82	0.96
SE	0.23	0.23	0.32	0.29	0.39	0.32	0.36

[a] r refers to the correlation between total solids measured gravimetrically and lactometrically.

[b] SE refers to the standard error of estimate.

[c] Not significantly different from zero at P = 0.05.

ing values of +0.01% and 0.36 are indicated for the small Watson lactometer (Tables 1 and 2). Under circumstances similar to those in the study reported here, 95% of the calculated total solids would be expected to fall within −0.01 ±0.72%, or from −0.73 to +0.71% total solids from the gravimetric values. At the average fat levels noted, an error of similar magnitude in fat test would be 0.24%.

Analyses of variance by breeds and for all breeds combined showed no significant differences between the lactometric and gravimetric methods for measuring total solids. Highly significant differences in total solids for fat levels were as expected. The absence of significant interactions between methods and fat levels indicated that different fat levels did not affect the relations between the two methods of measurement. The error mean squares ranged from 0.25 to 0.33.

These data indicate that the small Watson lactometer is useful for measuring the total solids of individual cows' milk. The value of the technique for field use is dependent on standard procedures, such as temperature control and reading techniques.

Small Quevenne lactometer. The results of the total solids measurements,

TABLE 3

Total solids measured by the small Quevenne lactometer and gravimetric methods, by breeds and fat levels (October, 1956, through June, 1957)

Breed	Fat levels (%)						
	<3	3	4	5	6	7	All
Holstein							
No. samples	13	508	226	12	.....	.....	759
Av. fat	2.7	3.5	4.3	5.2	.....	.....	3.8
Av. T.S.(G)[a]	11.22	12.14	13.17	14.21	.....	.....	12.47
Av. T.S. (L)[a]	11.40	12.21[b]	13.19	14.29	.....	.....	12.52
Guernsey							
No. samples	1	18	198	107	32	2	358
Av. fat	2.4	3.7	4.4	5.3	6.3	7.4	4.8
Av. T.S. (G)	10.43	12.40	13.56	14.68	15.86	16.95	14.05
Av. T.S. (L)	10.49	12.51	13.51	14.62	15.78	16.42[b]	14.00
Jersey							
No samples	.....	1	49	153	95	14	312
Av. fat	.....	3.9	4.6	5.5	6.3	7.2	5.7
Av. T.S. (G)	.....	11.22	13.96	15.09	16.13	17.11	15.30
Av. T.S. (L)	.....	11.72	13.94	15.00	15.96[b]	16.85	15.20
Brown Swiss							
No. samples	1	60	94	21	3	.....	179
Av. fat	2.8	3.7	4.4	5.2	6.3	.....	4.3
Av. T.S. (G)	11.26	12.49	13.72	14.96	16.07	.....	13.48
Av. T.S. (L)	11.35	12.50	13.67	14.81	15.60	.....	13.43
Ayrshire							
No.samples	.....	22	25	2	.....	.....	49
Av. fat	.....	3.6	4.4	5.0	.....	.....	4.1
Av. T.S. (G)	.....	12.56	13.38	14.13	.....	.....	13.04
Av. T.S. (L)	.....	12.56	13.35	14.09	.....	.....	13.03
All Breeds							
No. samples	15	609	592	295	130	16	1,657
Av. fat	2.7	3.6	4.4	5.4	6.3	7.2	4.4
Av. T.S. (G)	11.17	12.20	13.47	14.89	16.06	17.08	13.47
Av. T.S. (L)	11.34	12.26[b]	13.44	14.82	15.91[b]	16.79	13.46

[a] Average T.S. (G) and (L) refer to average total solids obtained by the gravimetric and lactometric methods, respectively.

[b] Significantly different from average total solids measured gravimetrically at P = 0.05.

using this lactometer and the gravimetric method, are presented (Tables 3 and 4). In several breed and fat groups, the average total solids measured by the lactometer were significantly different from those measured gravimetrically. The direction of the differences indicated that total solids measured lactometrically were higher than the gravimetric measurements at the low fat level (Holsteins and "All Breeds" at 3% fat) and lower at the high fat levels (Jerseys, Guernseys, and "All Breeds" at the 6 and 7% fat levels). Several of the significant differences were in groups with numerous observations, which indicated a real defect in the ability of this lactometer and/or the estimation equation to measure total solids.

All correlations presented (Table 4) were significant statistically, except for Brown Swiss samples at the 6% fat level. These correlations ranged from −0.13 to 1.00 for fat levels within breeds, from 0.91 to 0.96 for breeds when fat levels were ignored, and from 0.58 to 0.88 for fat levels when breeds were ignored. A correlation of 0.97 was obtained when fat levels and breeds were ignored. The corresponding standard errors of estimate ranged from 0.18 to 0.45, from 0.24 to 0.38, and from 0.20 to 0.35, respectively. A standard error of estimate of 0.32 was obtained when breeds and fat levels were ignored.

Analyses of variance by breeds and for all breeds combined showed significant differences between the lactometric and gravimetric methods for measuring total solids in the Holstein and Jersey groups. Again, highly significant differences between fat levels indicated that total solids of milk differed with fat levels. A significant interaction obtained between methods and fat levels for the data for all breeds combined indicated that the relations between the two methods differed

TABLE 4

Correlations and standard errors between total solids measured gravimetrically and with the small Quevenne lactometer, by breeds and fat levels (October, 1956, through June, 1957)

Breed	Fat levels (%)						
	<3	3	4	5	6	7	All
Holstein							
r[a]	0.83	0.88	0.85	0.80	.....	.....	0.93
SE[b]	0.22	0.19	0.24	0.20	.....	.....	0.25
Guernsey							
r	.....	0.80	0.73	0.86	0.40	1.00	0.92
SE	.....	0.32	0.30	0.22	0.24	.....	0.37
Jersey							
r	.....	.....	0.79	0.69	0.61	0.81	0.91
SE	.....	.....	0.29	0.32	0.37	0.25	0.35
Brown Swiss							
r	.....	0.91	0.84	0.80	−0.13[c]	.....	0.96
SE	.....	0.18	0.25	0.22	0.45	.....	0.27
Ayrshire							
r	.....	0.86	0.85	1.00	.....	.....	0.88
SE	.....	0.23	0.22	.....	.....	.....	0.24
All Breeds							
r	0.87	0.88	0.83	0.80	0.58	0.80	0.97
SE	0.22	0.20	0.28	0.28	0.35	0.28	0.32

[a] r refers to the correlation between total solids measured gravimetrically and lactometrically.

[b] SE refers to the standard error of estimate.

[c] Not significantly different from zero at $P = 0.05$.

at various fat levels. This interaction illustrates the fact noted above, that total solids measured by the small Quevenne lactometer were higher at the low fat level and lower at the high fat level than the gravimetric measurements. The error mean squares ranged from 0.22 to 0.30.

These data indicate that this lactometer and/or the estimation equation are not as satisfactory as the Watson lactometer for measuring total solids of individual cows' milk.

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HORMONAL CONTROL OF MAMMARY GROWTH AND LACTATION IN THE GOAT [1]

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SUMMARY

Mammary growth and lactation were induced in kids and mature goats by weekly injections of macrocrystalline stilbestrol alone, or of macrocrystalline progesterone and stilbestrol in combination at ratios of 40:1, 80:1, and 1,000:1. Sexually mature goats were distinctly more responsive to the treatments than 8-mo.-old goats. Although all groups reached approximately the same peak production 7 mo. from the start of the experiment, a more rapid onset of lactation was noted in those groups where progesterone and stilbestrol were combined in the treatment. Two goats approached closely the level of production recorded in the subsequent normal lactation. However, most of them were considerably below this level. No clear-cut indications were found for an optimum ratio between progesterone and estrogen for the induction of mammary growth and lactation in the goat.

It has been amply demonstrated that both estrogen and progesterone are required for normal development of the mammary glands of most of the laboratory and domestic animals that have been investigated to date. It is only in the guinea pig and monkey that complete development is obtained with estrogen alone.

Although limited mammary growth, together with varying amounts of milk secretion, can be obtained in the goat *(3, 4, 7)* and in the cow *(6, 17)* with estrogen alone, most workers agree that combined treatment with estrogen and progesterone is essential for normal development of the mammary lobule-alveolar system. Mixner and Turner *(11)* demonstrated that the alveolar epithelium of goats was more nearly normal when progesterone and estrogen were administered than when estrogen alone was administered. Similar results were reported by Sykes and Wrenn *(15)*, in heifer calves.

An optimal ratio between progesterone and estrogen for the induction of mammary growth has been reported for the rat *(14)*, mouse *(11)*, and rabbit *(8)*. However, Cowie *et al. (2)* reported that progesterone and estrogen given in ratios of 4:1, 40:1, 160:1, and 400:1 produced no higher milk yields than hexoestrol only (0.25–1.0 mg. daily). Reineke *et al. (13)* reported that in dairy cows, carrying implanted progesterone: stilbestrol pellets for from 88 to 124 days, followed by removal of the tablet residues and treatment with estrogen alone, satisfactory lactation was obtained with progesterone: estrogen ratios varying from 2:1 to 30:1. On the other hand, Turner *et al. (16)* reported recently that in cows injected daily with 100 mg. progesterone and 100 γ of estradiol benzoate (1,000:1 ratio) daily for 180 days, milk production rather closely approached the normal in a few of the treated animals.

Received for publication September 17, 1957.

[1] Published with the approval of the Director, Michigan Agricultural Experiment Station as Journal Series No. 2206.

In view of the rather wide diversity, both in methods of treatment and in the results reported, a study was undertaken in goats to compare the effectiveness for inducing lactation of stilbestrol and progesterone-stilbestrol combinations in several different ratios that were varied over a wide range. To circumvent the necessity of pellet implantation, or daily injection of an oil solution, the hormones were prepared as a macrocrystalline aqueous suspension that has a sustained effect and yet is well absorbed.

METHODS AND PROCEDURES

Eighteen dairy goats including Rock Alpines, Saanens, Toggenburgs, and Crossbreds were divided into four treatment groups that were matched as nearly as possible for age and breed (Table 1).

TABLE 1
Experimental design

Group	Goat No.	Breed	Age when experiment started	Stilbestrol	Progesterone
				(weekly)	
I	4	Saanen	2 yr.	1.75 mg.(11 wk.)	None
	19	Toggenburg-Nubian	8 mo.	1.75 mg.(11 wk.)	None
	7[a]	Nubian	4 yr.	1.75 mg.(11 wk.)	None
	16	Rock Alpine	8 mo.	1.75 mg.(11 wk.)	None
II	6	Toggenburg-Nubian	5 yr.	1.75 mg.(11 wk.)	70 mg.(9 wk.)
	18	Saanen	8 mo.	1.75 mg.(11 wk.)	70 mg.(9 wk.)
	8	Nubian	4 yr.	1.75 mg.(11 wk.)	70 mg.(9 wk.)
	21	Saanen	8 mo.	1.75 mg.(11 wk.)	70 mg.(9 wk.)
III	14	Rock Alpine	1 yr. 8 mo.	0.875 mg.(11 wk.)	70 mg.(9 wk.)
	24	Saanen	8 mo.	0.875 mg.(11 wk.)	70 mg.(9 wk.)
	15	Rock Alpine	1 yr. 8 mo.	0.875 mg.(11 wk.)	70 mg.(9 wk.)
	5	Saanen	8 mo.	0.875 mg.(11 wk.)	70 mg.(9 wk.)
IV	9	Toggenburg-R. Alp.	5 yr.	0.07 mg.(11 wk.)	70 mg.(9 wk.)
	22[a]	Saanen	8 mo.	0.07 mg.(11 wk.)	70 mg.(9 wk.)
	12	Nubian	2 yr.	0.07 mg.(11 wk.)	70 mg.(9 wk.)
	23[a]	Saanen	8 mo.	0.07 mg.(11 wk.)	70 mg.(9 wk.)
	10	Saanen-R. Alp.	1 yr. 8 mo.	0.07 mg.(11 wk.)	70 mg.(9 wk.)
	11	Toggenburg	1 yr. 8 mo.	0.07 mg.(11 wk.)	70 mg.(9 wk.)

[a] Animal not responding to initial treatment.

The macrocrystalline steroids to be administered were ground in a Potter-Elvehjem homogenizer with a 0.3% starch emulsion, a preparation which supplies a prolonged exposure of approximately 2 wk. [Nellor and Cole *(12)*]. The amount of progesterone administered (70 mg. per week) was the lower limit of the amount necessary *(10)* to maintain pregnancy in ovariectomized goats. The duration of treatment (9–11 wk.) corresponds to approximately one-half of the normal gestation period in the goat.

Two weekly injections of stilbestrol terminated each series of weekly injections on the supposition that this would facilitate the release of lactogenic hormone from the pituitary, as suggested by earlier work of Meites *et al. (9)*. Benson *et al. (1)* reported that estrogen priming, subsequent to progesterone-

estrogen treatment, had no beneficial effects on induced lactations in dairy goats. Although the progesterone-estrogen ratios in their study were wide, the amount of estrogen used was high. This might preclude the use of an estrogen primer, because sufficient estrogen may already have been present in the circulation.

Group I (Table 1) received 1.75 mg. of stilbestrol weekly for 11 wk. Group II received 1.75 mg. of stilbestrol and 70 mg. of progesterone; Group III received 0.875 mg. stilbestrol and 70 mg. progesterone, and Group IV received 0.07 mg. of stilbestrol and 70 mg. of progesterone, in a single subcutaneous injection once weekly for 9 wk. This represents a progesterone-estrogen empirical ratio of 40:1, 80:1, and 1,000:1, respectively. Groups II, III, and IV received two additional injections of 1.75 mg. of stilbestrol during the tenth and 11th wk. of the trial. The initial injection was given November 18, 1955.

Eight does were retreated 3 mo. from the start of lactation, to determine the effects of additional hormone therapy on animals failing to respond to the initial treatment and also on the existing lactation of does that had responded to the treatment.

Twice-a-day milking was initiated in Groups I and II when udder distention was noted during the tenth wk. of treatment, and in Groups III and IV one wk. later, one day before the last stilbestrol injection.

The goats were fed second-cutting alfalfa hay ad libitum, received 1.5 lb. of concentrate (16.0% protein) daily during the milking period, and were placed on pasture 10 wk. from the start of milking. Twice-a-day milking was continued for 33 wk. unless the doe did not respond to the treatment, in which case milking was discontinued. Milking was terminated on October 15, 1956, in all does still lactating at that time.

The level of stilbestrol injected did not appear excessive, since only one of the stilbestrol-treated does (No. 16) showed estrus during the treatment. Slight vulvar stimulation was detected in all treated groups by the end of the 3rd wk. of treatment, a phenomenon more evident in the kids than in the aged animals. Weekly palpation of the udder region revealed some development of the udder pad of all treated groups as early as the end of the 4th wk. of treatment, although comparison of the degree of development was not feasible by this method.

The presence of a serous fluid was noted in the teats of a few goats from each group by the 5th wk. of treatment, but obvious distention of the gland with fluids was not apparent in the 80:1 or 1,000:1 groups during the combined progesterone-estrogen treatment.

The most distinct difference in lactational response is found in comparing Groups I (stilbestrol only) with the three groups receiving progesterone-stilbestrol combinations (Figure 1). Only three of the four animals in Group I came into lactation. Peak production was reached later and was generally at a lower level than in the other groups. This is in accord with earlier findings *(11)*, that both progesterone and estrogen are needed for normal mammary growth. However, in comparing the results for Groups II, III, and IV, little evidence is found for an optimal ratio between progesterone and estrogen at the levels and

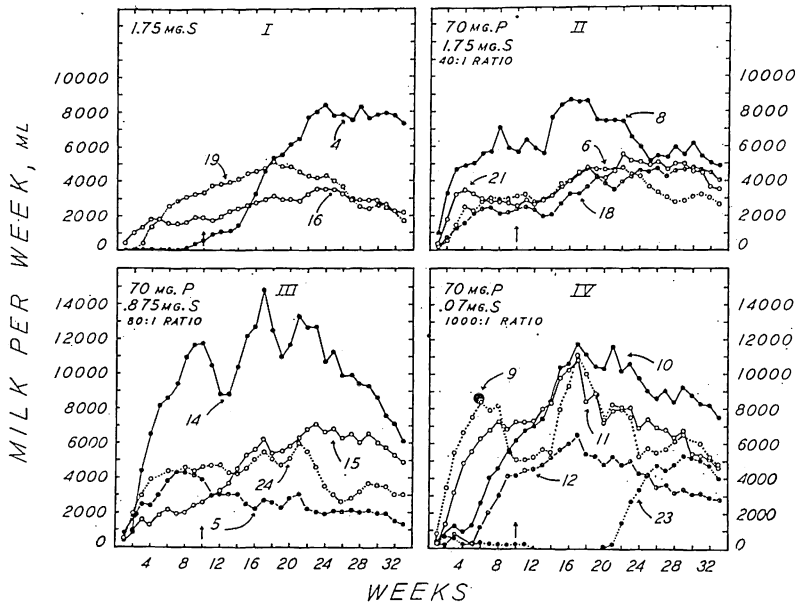


FIG. 1. Weekly milk production induced by hormonal treatment.

in the proportions employed in these trials. All animals in Groups II and III came into lactation. Goat No. 14 (Group III) had the highest production in the entire experiment, yet the remaining animals in this group have records very similar to the three lowest producers in Group II. In the first three groups all of the 8-mo.-old kids responded to the initial treatment with some degree of lactation. In Group IV only the four sexually mature goats responded, no appreciable lactation being obtained in the 8-mo.-old kids. Consequently, the tendency toward higher milk production in Group IV as a whole can probably be accounted for by the disparity in ages.

Retreatment of three of the goats that failed to respond initially (No. 7, Group I, and No. 22 and 23, Group IV) with 0.875 mg. of stilbestrol and 70 mg. of progesterone weekly for 7 wk., resulted in only one doe (No. 23) responding and reaching a production level comparable to that of other responsive animals. Similar retreatment of does already in lactation (No. 16, Group I; No. 6 and 21, Group II, and No. 5 and 15, Group III) caused no apparent inhibition or enhancement of milk production, either during the retreatment or subsequent to it.

In Table 2, the milk yields for the experimental lactations of 12 of the goats that kidded the following year are compared with their normal lactations. Age corrections have not been employed, because factors are not available that would apply to our conditions. From this comparison, it is again apparent that sexually immature animals are distinctly less responsive to the treatments given than are mature goats. Five kids that were approximately 8 mo. of age at the beginning of treatment had 6-mo. experimental milk yields ranging from 20.3 to 54.5% of their normal, and averaged 33%. Seven older animals averaged 62.1% of normal, ranging from 48.8 to 86.3%.

TABLE 2

Induced versus normal [a] milk production

Group	Goat No.	Age Yr.	Age Mo.	Max. monthly production: Induced Month	Max. monthly production: Induced Pounds	Max. monthly production: Normal Month	Max. monthly production: Normal Pounds	Max. monthly production: Normal (%)	6-month production (183 days) [b]: Induced Pounds	6-month production (183 days) [b]: Normal Pounds	6-month production (183 days) [b]: Normal (%)
	4	2		8	77.9	3	130.5	59.7	361.7	616.7	58.7
I	19		8	5	47.0	3	215.8	21.8	220.4	889.3	24.8
	16		8	6	33.1	2	144.9	22.8	159.8	612.9	26.1
II	18		8	8	47.0	2	123.0	38.2	217.3	550.7	39.5
	8	4		5	97.2	2	117.9	82.4	408.2	554.8	73.6
	14	1	8	5	126.4	2	169.3	74.7	640.9	742.7	86.3
III	24		8	5	53.0	2	123.3	43.0	259.7	476.5	54.5
	15	1	8	5	65.9	2	136.1	48.4	305.4	626.3	48.8
	5		8	2	41.3	2	160.4	25.7	169.7	834.4	20.3
	12	2		4	57.2	2	167.7	34.1	259.0	621.0	41.7
IV	23		8	5	49.7	2	122.8	40.5	.....	.....	.....
	10	1	8	5	107.6	2	125.3	85.9	517.7	600.2	86.3
	11	1	8	4	92.2	2	149.2	61.8	446.0	692.7	64.4

[a] Obtained from subsequent lactation following pregnancy. Animals not included were not bred following the induced lactation.
[b] Six successive months of highest production.

Two of these animals (No. 14 and No. 10) approached their normal lactations closely, when the difference in age between successive records is considered. However, they were from two different groups, receiving widely different ratios of progesterone to stilbestrol. This raises the question whether a dosage level and combination can be found that will be optimal for all animals, or whether there is too much variability in hormone requirement and responsiveness between animals for this approach to be feasible. It will be necessary to test further hormone combinations and dosage levels before this question can be answered.

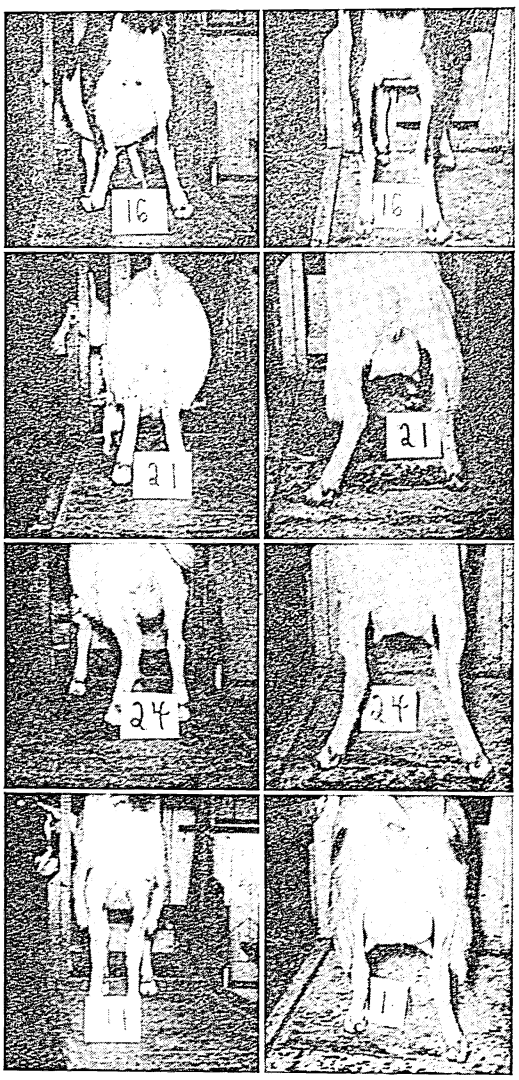


FIG. 2. Mammary development induced by hormonal treatment. Photographs at left at time of initial treatment. Photographs at right 12 wk. later. No. 16, 1.75 mg. stilbestrol per week; No. 21, 24, and 11 each received 70 mg. of progesterone weekly, and 1.75, 0.875, and 0.07 mg. stilbestrol, respectively.

It is of interest to compare visually the udder development in those does not having had a lactation previous to treatment, although it is recognized that this type of comparison has serious limitations. The gross udder development in kids No. 16, 21, and 24 and a yearling doe, No. 11 (Figure 2), is typical of their respective groups.

ACKNOWLEDGMENT

The authors are indebted to Mr. Horst Kaczkofsky for technical assistance with the experiments reported herein.

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INFLUENCE OF STAGE OF MATURITY AND OF GROUND SNAP CORN OR SODIUM METABISULFITE AS PRESERVATIVES ON THE FEEDING VALUE OF OAT SILAGE [1]

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SUMMARY

Fall-sown oats, harvested and stored in 20-ton upright silos, were evaluated with milking cows. In 1956, two silos were filled with oats cut at the prebloom stage, one at milk and one at dough stage. The 1957 silages included two silages cut at the boot stage, two cut at the prebloom stage, and one cut at the milk stage. Ground snap corn and sodium metabisulfite were compared as preservatives. Optimum dry matter preservation was achieved with the ground snap corn, with oats cut at the prebloom stage. In six silos where direct comparison was possible, the ground snap corn preserved 13% more dry matter than the sodium metabisulfite. Dry matter intake was only slightly influenced by the preservative and increased with stage of maturity through the milk stage in 1956 and prebloom stage in 1957. Persistency of milk production over a 28-day period was used as the criteria of feeding value. Only the oats cut at the boot stage were satisfactory for milk production when preserved with sodium metabisulfite, but all silages preserved with ground snap corn resulted in normal milk production.

In recent years, there has been a surge of interest in oat silage as a feed for dairy cows. This interest has resulted from low yields of corn during dry summers and a need for a spring-harvested silage for summer feeding. Few references were found in the literature relative to the influence of stage of maturity, and none on the value of ground snap corn and sodium metabisulfite as preservatives for oat silage.

Meyer *et al. (5)* harvested oat forage from the same field at seven stages of maturity and fed the hay to growing sheep. The highest nutritive value was found in the jointing stage, but the greatest production of nutrients per acre was from the 18% flower stage.

Thurman *et al. (6)* harvested oats at six stages of maturity and determined digestibility coefficients with steers. They concluded that oats should be cut in the boot-to-milk stage for high-quality silage.

Bratzler *et al. (2)* reported marked improvement in nutrient preservation from the use of sodium metabisulfite. Variability between control silages was observed by Gordon *et al. (4)* to be the primary factor influencing the apparent benefit obtained from preservatives. Gordon *et al. (3)* and Bohstedt *et al. (1)* reported improved nutrient preservation with corn meal.

EXPERIMENTAL PROCEDURE

Fall-sown oats were harvested at various stages of maturity and stored in 20-ton upright silos. Each load of silage was weighed before unloading, and

Received for publication December 31, 1957.

[1] Georgia Experiment Station Journal Series, N. S. 327.

the proper amount of preservative was carefully weighed and added. Eight pounds of sodium metabisulfite or 200 pounds of ground snap corn was used per ton of fresh oat forage. The ground snap corn was spread over the silage on the truck and the sodium metabisulfite was added at the blower. Dates of cutting, yield-per-acre, dry matter content, and maximum temperature recorded at the center of each silo are presented (Table 1). The silage cut in 1956 was from an excellent oat crop, whereas the 1957 silage represents a year when crown rust damaged the crop.

In 1956, the three stages at which oat forage was ensiled were the prebloom, milk, and dough; in 1957, the three stages were the boot, prebloom, and milk. For convenience, the silages were numbered consecutively; the last number indicates year of harvest.

Silages 4–6 and 5–6, 6–7 and 7–7, and 9–7 and 10–7 were made by filling silos with alternate loads of forage. All other silages were harvested as single silos. To simulate farm conditions, each silo was filled at the approximate rate of filling expected in making silage in 100-ton silos.

The silos were opened and silages fed as needed. The silage was weighed out of the silo and samples were collected three times each week for dry matter, pH, and other analysis. Samples were frozen, and composited when the silo was empty, for the usual "proximate" analysis. Analytical methods were those recommended at a conference of silage investigators *(6)*.

Feeding value of the silages was evaluated in 28-day feeding trials with four or five milking cows. The cows were fed silage free choice and grain was fed at about 1:6. In some instances, it was necessary to feed cottonseed meal because of the low protein content of the silage. Hay was fed with the first two silos, but was discontinued to enable the remaining silos to be fed according to recommendations of the silage conference *(6)* referred to above. Silages 1–6 and 8–7 are included for reference only. Silage 1–6 was fed with temporary winter

TABLE 1

Date of cutting, yield per acre, dry matter content, and maximum silo temperature of the silages

Stage of maturity and silage No.	Date of cutting	Yield per acre	Dry matter of forage	Maximum temperature in silo
		(tons)	*(%)*	*(° F.)*
Dough				
1–6[a]	5–9–56	15.0	32	85
Milk				
2–6	5–1–56	16.0	29	84
3–7	4–29–57	8.0	35	94
Prebloom				
4–6	4–20–56	15.0	25	78
5–6	4–20–56	15.0	25	90
6–7	4–24–57	9.5	28	95
7–7	4–24–57	9.5	28	100
8–7	4–22–57	9.0	24	100
Boot				
9–7	4–10–57	9.7	24	72
10–7	4–10–57	9.7	24	85

[a] Second number indicates year in which silage was made.

pasture, and Silage 8–7 was stored in a trench silo, but represents an untreated silage. Milk-production persistency during the 28-day feeding trial was considered the chief criteria of feeding value.

RESULTS AND DISCUSSION

Preservation of nutrients. The composition of the silages is shown in Table 2, and the preservation of nutrients, in Table 3. On the basis of physical characteristics such as odor, color, fermentation type, and pH, all of the silages would have been scored as good to excellent on the score-cards suggested by the silage conference *(6)*. (Stage of maturity was ignored in scoring, because it was one of the factors under study.) With the exception of Silages 4–6 and 9–7, the temperatures were within the optimum range of 80–100° F. In general, the

TABLE 2

Chemical composition of the silages (dry matter basis)

Stage of maturity and preservative	Silage No.	Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	pH	Dry matter
		(%)						
Dough								
$Na_2S_2O_5$	1–6[a]	7.58	4.22	35.02	47.33	5.85	4.1	27
Milk								
$Na_2S_2O_5$	2–6	9.20	5.66	20.90	46.94	7.30	4.1	27
None	3–7	8.65	2.94	36.99	46.26	5.16	4.0	29
Prebloom								
$Na_2S_2O_5$	4–6	10.71	3.76	29.11	47.27	9.15	4.3	25
Snap Corn	5–6	9.31	8.03	23.59	54.41	4.66	3.7	30
$Na_2S_2O_5$	6–7	10.12	3.24	36.36	44.45	5.83	4.1	25
Snap Corn	7–7	10.30	3.59	29.87	52.06	4.18	3.9	29
None	8–7	8.24	2.92	37.56	45.94	5.34	3.8	24
Boot								
$Na_2S_2O_5$	9–7	10.47	4.38	35.58	43.43	6.14	4.1	19
Snap Corn	10–7	11.93	4.23	27.00	51.55	5.29	3.8	28

[a] Second number indicates year in which silage was made.

chemical composition followed the expected changes with stage of maturity. Crude protein was lower than desired, although all silages were higher in crude protein in the 1957 crop than in the 1956 crop. The lower per-acre yields may have influenced this fraction. The usual changes in chemical composition—increases in crude fiber and ether extract and a decrease in NFE—were observed. Despite the care taken in weighing the forage and silage, there were occasional small losses due to wind and accidents. It was felt, however, that the forage losses were less than 100 lb. per silo.

The data (Table 3) also follow a general pattern relative to preservation of total nutrients. Within a preservative treatment, dry matter preservation increased with increasing stage of maturity until the dough stage. The lower percentage of preservation in the dough stage was due to heavy losses from spoilage resulting from poor compaction. This loss took the form of rotted silage near the walls of the silo and was difficult to weigh accurately, because the exact point of separation between good and spoiled silage was not clear. The

TABLE 3

Per cent of stored nutrients preserved as good silage.

Stage of maturity and preservative	Silage No.	Per cent of stored constituents preserved						
		Fresh material	Dry matter	Crude protein	Ether extract	Crude fiber	N.F.E.	Ash
		(%)						
Dough								
$Na_2S_2O_5$	1–6[a]	91.6	76.8	83.0	101.6	86.4	66.9	100.0
Milk								
$Na_2S_2O_5$	2–6	88.8	82.5	96.5	157.2	83.6	71.6	133.5
None	3–7	95.5	79.0	88.7	104.8	90.4	68.8	77.4
Prebloom								
$Na_2S_2O_5$	4–6	85.7	86.4	108.5	116.0	98.2	70.6	150.0
Snap Corn	5–6	87.3	94.0	100.8	263.0	103.5	81.6	99.5
$Na_2S_2O_5$	6–7	84.2	75.9	74.2	109.5	90.9	64.3	97.4
Snap Corn	7–7	89.6	84.9	88.3	130.8	87.7	81.2	80.1
None	8–7	.....	.....	.....	.....	.....	.....	.....
Boot								
$Na_2S_2O_5$	9–7	69.4	54.3	55.5	114.8	85.5	39.4	62.6
Snap Corn	10–7	72.4	76.0	87.0	140.5	92.3	65.5	76.5
Av. of silages preserved with:								
Snap Corn		83.1	85.0	92.0	178.1	94.5	76.1	85.4
$Na_2S_2O_5$		79.8	72.2	79.4	113.4	91.5	58.1	103.3

[a] Second number indicates year in which silage was made.

general influence of the two preservatives was quite pronounced. When Silages 4–6, 6–7, and 9–7 are averaged and compared with the mean of Silages 5–6, 7–7, and 10–7, the ground snap corn preserved 13% more dry matter than the sodium metabisulfite. This additional preservation was represented in all fractions except ash, and was most evident in ether extract and N.F.E., indicating a better preservation of the carbohydrates.

Such a preservation may have a marked influence on the usefulness of a preservative. To determine if this difference in preservatives is of real significance, a comparison of the efficiency of dry matter preservation is presented (Table 4). Using the data for the silage preserved with sodium metabisulfite as a basis for comparison, the table indicates that, on the average, for each 15 lb.

TABLE 4

Stored losses of forage and additives

	Material	Dry matter	Storage loss	Recovered dry matter
	(lb.)			
Forage stored with $Na_2S_2O_5$ and snap corn stored in a bin:				
Forage	2,000	460	138	322
Snap Corn	200	180	0	180
Forage stored with added snap corn:				
Forage	2,000	460	69	391
Snap Corn	200	180	15	165
Net gain of treatment:				
Forage dry matter		−69		
Snap Corn		−15		

of snap corn lost during silage fermentation there was a net gain of 69 lb. of forage dry matter in the silos preserved with ground snap corn. This increase in preservation may or may not be of economic importance, depending upon the relative value of ground snap corn and silage dry matter. The comparison does not necessarily reflect the true differences, because it does not evaluate the relative feeding value of the two resulting rations.

Feeding value of the silages. The objectives of the feeding trials were (1) to determine which of the silages was most suitable for dairy cows and (2) to create differences in response wide enough to differentiate between stages of maturity and preservatives. This dual function of the feeding trial created problems which were not entirely overcome in this experiment. In attempting to feed against obvious protein deficiencies due to stage of maturity, actual differences may have been narrowed. The feeding trial data are also complicated by differences in total dry matter intake which resulted from free-choice feeding of the silage. Earlier feeding trials using oat silage had indicated the silage to be a low-energy feed; for this reason, no change in the grain fed was made with the ground snap corn silages. Once started, this feeding plan was continued throughout the trials. Intake and production data are shown (Table 5).

In general, there was no consistent difference in silage intake due to preservative. The difference in silage dry matter resulted in a mean difference in dry matter intake of 4 lb. per cow per day in favor of the snap corn. In general, silage consumption increased with increasing stage of maturity. This increase continued through the milk stage in 1956 and through the prebloom stage in 1957. The consumption and utilization of the milk-stage oats illustrate the variation which may be encountered between years. Silage 3–7 was ensiled in 1957, which was a very poor year for oats. The crop had suffered heavy disease damage and, by the time it was in the milk stage, heavy leaf damage had occurred.

TABLE 5

Rations fed and animal response to the silages

Stage of maturity and preservative	Silage No.	Av. daily intake per cow				Silage per 1,000 lb. live weight	Beginning milk production	28-day change	
		Silage	Hay	Grain	Total dry matter			FCM	Body weight
		(lb)						(%)	(lb.)
Dough									
$Na_2S_2O_5$	1–6	50.4	0	5.4	26.0[b]	50	26.2	−10	−12
Milk									
$Na_2S_2O_5$	2–6	89.4	0	5.0	27.5	88	26.3	−12	+10
None	3–7	52.0	0	6.1	20.4	59	24.3	−30	−16
Prebloom									
$Na_2S_2O_5$	4–6	75.6	8.0	9.0[a]	34.0	72	27.6	−22	− 8
Snap Corn	5–6	82.3	8.0	7.8[a]	37.9	77	23.1	− 6	+10
$Na_2S_2O_5$	6–7	84.5	0	4.7	25.3	98	27.8	−26	+23
Snap Corn	7–7	80.6	0	4.7	26.8	96	28.3	− 9	+35
None	8–7	83.1	0	7.0	27.1	87	27.5	− 8	−15
Boot									
$Na_2S_2O_5$	9–7	66.4	0	4.3	16.5	71	25.9	−10	− 1
Snap Corn	10–7	66.7	0	4.3	22.5	71	25.6	− 9	+ 1

[a] Includes 2 lb. cottonseed meal.
[b] Two hours of grazing on winter oats.
[c] Second number indicates year in which silage was made.

The estimated grain yield would have been cut by at least 50%. The prebloom oats showed much less variation and this factor may be of considerable importance in deciding the stage to cut oats, particularly in a year such as 1957. The cows consumed sufficient nutrients on all silages to meet accepted feeding standards, but animal response would seem to indicate that factors other than TDN and digestible protein were involved.

Animal response as measured by milk production was satisfactory on all silages preserved with ground snap corn, but only the oats ensiled at the boot stage resulted in satisfactory milk production when preserved with sodium metabisulfite. The one comparison with oats ensiled without preservative (Silage 8–7) indicated that sodium metabisulfite resulted in a less valuable silage than a well-made silage without preservative. This comparison brings out a factor of considerable importance to silage experiments, because the value of a preservative depends upon the value of the control silage. Had the silage without preservative been poorly fermented, the comparison between the silages could have been quite different.

Stage of maturity had little influence on milk production, so long as the forage was cut prior to the milk stage. The milk-stage silage failed to support a normal rate of milk production both years. The one silage cut at the dough stage was unable to support normal milk production, although fed with high-quality pasture.

When both milk production and preservation of nutrients were considered, the optimum stage for cutting oats was in the prebloom stage.

CONCLUSIONS

Ten silos were used to compare stage of maturity and two preservatives for oat silage. The following conclusions are valid: Optimum preservation of nutrients and feeding value was obtained at the prebloom stage of maturity. The use of 200 lb. per ton of ground snap corn improved nutrient preservation and resulted in silage of acceptable feeding value. Sodium metabisulfite was an unsatisfactory preservative for oat silage. The value of this preservative under conditions of poor silage-making was not studied.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Margaret Borders for aid in the laboratory, to Harold Childres for supervising the feeding trials, and to H. S. Johnson, Jr., for analysis of the silages.

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BORON IN COWS' MILK[1]

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SUMMARY

Boron appears to be a natural constituent of cows' milk, the amount varying with the intake of the element in the feed. Three hundred milligrams of boron fed daily as sodium tetraborate for a period of 2 mo. to six cows increased the average amount of boron in the milk approximately 2.5 times, from 270 to 663 μg. per liter of milk. The total daily amount of boron thus secreted appeared to be rather constant for individual cows, regardless of the amount of milk produced. A consistent increase in boron content of the milk was noted from the 1st to the 2nd mo. in each half of the trial, regardless of breed, group, or treatment. No plausible explanation for this increase fits the situation as a whole. A study of partition of the boron between cream and skimmilk indicated that about one-fourth of the total amount was in the cream and three-fourths in the skimmilk.

Boron is an essential element for plants and is added to fertilizers for specific crops in certain areas, especially where agriculture is highly intensified. Alfalfa and sainfoin *(10)*, soybean meal, and beet pulp *(7)*, in comparison with some common grasses *(6, 9)*, contain considerable amounts of boron. Previous investigators *(1)* have shown that boron is widely distributed in animal tissues.

Several earlier investigators *(2, 3, 4, 13)* detected its presence spectrographically in the ash of milk, but did not determine the amount. More recently Hove *et al. (8)* reported values ranging from 94 to 228 μg. per liter of milk; they concluded that the amount is influenced by the boron content of the feed. Owen *(12)* found that the addition of 20 g. of borax to the daily ration of milk cows increased the boron content of the milk from 0.7 to over 3.0 p.p.m.

EXPERIMENTAL PROCEDURE

This work was done during the winter of 1956–57 with six cows divided into two groups of three each, representing the Ayrshire, Guernsey, and Holstein breeds. Each breed pair was matched as closely as possible with respect to stage of lactation. The supplement fed was borax ($Na_2B_4O_7 \cdot 10\ H_2O$) in a daily amount of 2.6 g., equivalent to 300 mg. of elemental boron. One group received the supplement during December and January, the other during February and March.

Composite two-day milk samples of one liter were taken from each cow about the middle of each month. Triplicate 300-ml. aliquots were placed in silica dishes, mixed with a slurry of 1 g. CaO and H_2O, and evaporated to dryness on a steam bath. The samples were then ashed in an electric furnace at approximately 600° C. and the ash dissolved in 6 *N* H_2SO_4. Boron was determined photometrically by the method of Oelschläger *(11)*. Reproducibility of the method was

Received for publication January 3, 1958.

[1] Contribution No. 1140.

determined by adding 100 and 200 μg. of boron to 300-ml. aliquots of normal herd milk. Percentage recoveries were 90, 95, 90, and 105; average, 95%.

RESULTS

Results are summarized (Table 1). The boron content when the cows were on the control ration averaged 270 μg. per liter; the range was from 193 to 360 μg/liter. When the supplement was being fed, the average value was 663 μg/liter, with a range from 510 to 860 μg/liter. The increase was highly significant. One cow (A105) did not show any increase when fed the supplement. No reason can be assigned for this; therefore, the values for this cow, for February and March, are not included in the average. The average level of boron in the milk when the supplement was fed was approximately 2.4 times that found when the cows were on the control ration.

TABLE 1

Effect of feeding cows sodium tetraborate on boron content of milk

	Cows on control ration				Cows receiving supplemental boron			
	First half of season							
	A105	G104[a]	H71	Average	A71	G100	H70[b]	Average
	(μg. boron/liter milk)							
December	260	267	243	261	677	593	510	593
January	360	.....	293	327	860	660	527	682
Average—first half	310	.....	268	285	769	627	519	638
	Second half of season							
	A71	G100	H39	Average	A105[c]	G86	H71	Average
	(μg. boron/liter milk)							
February	260	260	193	238	243	743	593	668
March	268	327	243	279	293	777	693	735
Average—second half	264	294	218	259	268	760	643	702
Average—entire season	287	281	241	270 ±13[d]	.....	693	581	663 ±35[d]

The initial letter prefixed to each cow's number indicates the breed.

[a] Through a misunderstanding, this cow was slaughtered before the January samples were taken; Cow G86 was substituted.

[b] Slaughtered because of repeated attacks of acute mastitis; Cow H39 was substituted in February.

[c] Not included in the average.

[d] Standard error of the mean.

The results from the control cows are of the same order of magnitude as those obtained by Falckenthal *(6)*; they are somewhat higher than Hove's values *(8)* (94–228 μg. per liter), possibly owing to the fact that considerable beet pulp was fed. This material has been shown *(7)* to be relatively high in boron.

The results when the supplement was fed are somewhat lower than those reported by Owen *(12)*, especially with respect to maximum value (860 μg. per liter in contrast to 3,000). However, Owen fed 20 g. of borax daily, or nearly

eight times as much as was fed in this investigation, confirming the conclusion of Hove *et al. (8)* that the amount of boron in milk is influenced by the amount in the feed.

The daily output of boron in the milk, based on the milk yield during the two-day sampling periods, is shown (Table 2). Individual cows, whether or not they were receiving the supplement, showed a rather constant output of boron in their milk.

TABLE 2

Boron output in the milk

(Based on average two-day milk yield for the several sampling periods)

	Cows on control ration			Cows on boron-supplemented ration		
	First half of season					
	A105	G104	H71	A71	G100	H70[b]
	(μg. boron daily)					
December	4.37	2.75	5.53	12.56	6.80	8.79
January	5.22	([a])	5.34	12.73	6.92	7.50
	Second half of season					
	A71	G100	H39	A105[c]	G86	H71
	(μg. boron daily)					
February	3.39	2.98	3.74	3.36	11.85	10.29
March	3.64	3.31	4.51	3.50	12.11	11.25

[a] See footnote [a] under Table 1.
[b] See footnote [b] under Table 1.
[c] Low values were due to the low level of boron in this cow's milk.

It is of interest also to note that without exception, and regardless of whether or not the cows were receiving the boron supplement, the values in each cow's milk for the second month of each half of the trial were somewhat higher than those for the first month. These increases ranged from 8 to 183 μg/liter, or from 3.1 to 38.5%; most of the increase can be explained on the basis of the results in Table 2 *(q.v.)*. The rather constant output of boron that accompanied the normal decrease in milk yield accounts largely for these higher values in the second month.

Partition of the boron between fat and solids-not-fat of the milk was also investigated. It was found that the boron content of cream (40% fat) was about 3.6 times higher than that of the skimmilk. On this basis, ordinary fluid milk containing 4% fat would have about one-quarter of the total amount of boron in the cream, the remaining three-quarters being in the skimmilk.

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EFFECT OF POTASSIUM NITRATE INTAKE ON DAIRY CALVES[1]

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SUMMARY

This study provides information on the effects of level, frequency, and duration of nitrate intake on the nitrate tolerance of young dairy animals. Administration of KNO_3 to bull calves indicated that the minimum lethal dose for initial intake was in excess of 30 g. per 100 lb. body weight. Toxic response to the compound was found to vary between animals, but was not materially affected by experimental method of intake (by capsule or dissolved in milk). The level of KNO_3 ingestion exerted little, if any, influence on the time required for the in vivo formation of maximal levels of methemoglobin (determined spectrophotometrically). Up to 1.3 g. % nitrate, but no nitrite, was detected by microbiological assay of the urine from two calves receiving 25 g. KNO_3 per 100 lb. body weight and showing low levels of methemoglobin.

During recent years, nitrate poisoning has become increasingly important to the livestock industry, either by virtue of an increased incidence or owing to more accurate diagnosis of the symptoms. Most of the early work indicates that this condition was associated primarily with the ingestion of oat hay or corn stalks high in KNO_3 equivalent *(2, 5, 9, 15–17)*. More recently, other feed materials such as corn silage, sorgo silage, and alfalfa hay also have been implicated in nitrate poisoning of cattle and sheep, when the KNO_3 equivalent on a dry matter basis exceeded approximately 0.7% of the feedstuff.

Davidson *et al. (5)* reported that the symptoms of nitrate poisoning were quite uniform in character, and that the severity of these symptoms was markedly increased if the animals were disturbed, or forced to move about. The symptoms included an increased pulse rate, weakness, staggering gait, apparent blindness in some cases, and cyanosis; in severe cases, death usually occurred with little or no struggling. Similar symptoms were reported by Muhrer *et al. (13)*, with the exception that motor spasms progressed rapidly to convulsions, terminating in death. However, in the latter report, nitrate was only one of a group of many factors believed to be responsible for the extreme toxicity of forages produced in a drought area. Beath *et al. (1)* have reported similar symptoms.

Ingested nitrates are believed to be converted to nitrites in the digestive tract *(1, 12)*, and the nitrites then absorbed by the blood stream cause the hemoglobin to be converted into methemoglobin, which is incapable of carrying oxygen to the tissues *(1, 6, 12, 13, 14)*. The methemoglobin will gradually be reduced to normal hemoglobin in drawn blood samples *(6, 10)*.

Relatively little information is available on the importance of nitrate poisoning in young animals and on its relationship to their later growth and development. However, Bradly *et al. (4)* report that the minimum lethal dose of KNO_3

Received for publication August 28, 1957.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 1787. Approved by the Director.

(by stomach tube) for dairy calves is approximately 25 g. per 100 lb. body weight, with large quantities of nitrite being found in the bile and urine of animals receiving the nitrates.

METHODS

Bull calves from the University of Missouri dairy herd were used as experimental animals. The calves nursed their dams for the first three days after birth and were bucket-fed thereafter, receiving whole milk (10% of body weight daily) to the age of 1 mo., followed by a gradual change to skimmilk at a daily rate of 10% of body weight, until a maximum of 14 lb. skimmilk was reached. Grain was fed free choice, with the older calves consuming approximately 4 lb. per day. Alfalfa hay, containing less than 0.21% KNO_3 equivalent on a dry matter basis, was fed ad libitum.

Potassium nitrate dosages were administered either by gelatin capsule or by dissolving the compound in skimmilk.

Blood samples were drawn from the jugular vein into sterile tubes containing 1.5 mg. potassium oxalate per milliliter of blood.

Analysis for hemoglobin and methemoglobin was started within 30 min. after the sample was drawn, since methemoglobin in stored blood is converted back slowly to functional hemoglobin *(10)*. A Coleman Model 14 Spectrophotometer was used with the method of Evelyn and Malloy *(7)*, for determining hemoglobin and methemoglobin.

Urine samples were collected from a limited number of calves, using a modification of the urine-collection apparatus as designed by Hobbs *et al. (11)*. Urine samples were held in a frozen state (−15° C.) between collection and analysis for nitrate and nitrite content. The microbiological method developed by Garner *et al. (8)* was used in determining the nitrate and nitrite content of the alfalfa hay and urine samples.

OBSERVATIONS AND RESULTS

Individual animals vary markedly in the time that maximum conversion of hemoglobin to methemoglobin occurs following nitrate ingestion (Table 1). However, neither the level of nitrate intake nor the mode of intake (gelatin capsules vs. milk) appears to have much influence on the time of maximum conversion. The slight increase in methemoglobin formation with KNO_3 given in capsules may be, in part, owing to a carryover in toxic effect from the dosage given in milk during the preceding week. Most of the calves refused to drink milk containing more than 1.9 g. % KNO_3.

In the experimental trials, maximum conversion occurred approximately 5 hr. after nitrate intake. In contrast to the 25-g. minimum lethal dose reported by Bradly *et al. (4)*, no calves died in this series of feeding trials, even though doses up to 30 g. per 100 lb. body weight were given. A possible explanation for the difference in tolerance could be owing in part to the level of energy in the ration and to the condition of the calves at the time of feeding potassium nitrate.

The response (conversion of hemoglobin to methemoglobin) of calves to initial KNO_3 intake shows considerable individual variation, but also exhibits a general trend of increase in toxic symptoms with increasing KNO_3 dosage levels (Table 1). The maximum methemoglobin values at 10, 15, 20, and 30 g. per 100 lb. body weight dosages were 0, 8, 16, and 37%, respectively.

In all cases where animals received nitrates, and showed methemoglobin formation, there was an increase in heart rate (Figure 1).

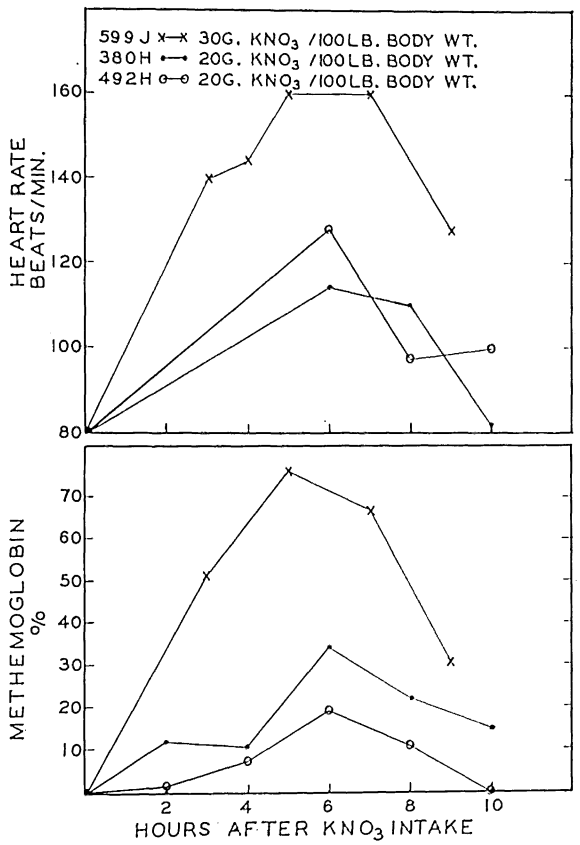


FIG. 1. Relationship between heart rate and methemoglobin formation.

Large quantities of nitrates were found to be excreted in the urine of calves receiving 25 g. KNO_3 per 100 lb. body weight (Figure 2). However, no nitrites were detected in the urine at this level of KNO_3 intake. As shown in Figure 2, excretion of nitrates in the urine, and conversion of hemoglobin to methemoglobin in blood, occur at approximately the same time following KNO_3 ingestion.

Two calves were given multiple doses of potassium nitrate (15 g. per 100 lb. body weight) over a period of eight days. The calf receiving a single daily KNO_3 dose gained an average of 2 lb. in body weight per day and showed less than 10% maximum conversion of hemoglobin to methemoglobin on any given day. The other calf, receiving a total of 30 g. per 100 lb. body weight daily, in two equal portions given 12 hr. apart, neither gained nor lost weight during the

TABLE 1

Time of maximum hemoglobin-to-methemoglobin conversion following potassium nitrate intake

Calf No.	Age	Body wt.	Dosage level	Method of KNO_3 intake	Maximum conversion HB to MHB	Hr. after intake when maximum conversion occurred
	(wk.)	*(lb.)*	*(g. KNO_3/cwt)*		*(%)*	
492H	6	155	10	Capsule	0	....
	9	200	20	Capsule	19	6
380H	5	147	10	Capsule	0	....
	8	190	20	Capsule	34	6
490H	8	188	15	Capsule	8	5
460H	7	168	15	Capsule	4	5
498H	8	146	20	Milk	0	....
	9	162	20	Capsule	0	....
	10	164	25	Milk	5	4
	11	181	25	Capsule	19	8
495H	9	158	20	Capsule	5	5
	10	174	20	Milk	1	7
	11	185	25	Milk	3	4
	12	200	25	Capsule	4	4, 6
485H	10	181	20	Capsule	16	3
	12	206	25	Milk	4	4
	13	223	25	Capsule	11	4
599J	15	172	30	Capsule	37	9
	18	198	30	Capsule	76	5
	20	224	25	Milk	3	4, 6, 8
	21	238	25	Capsule	3	4

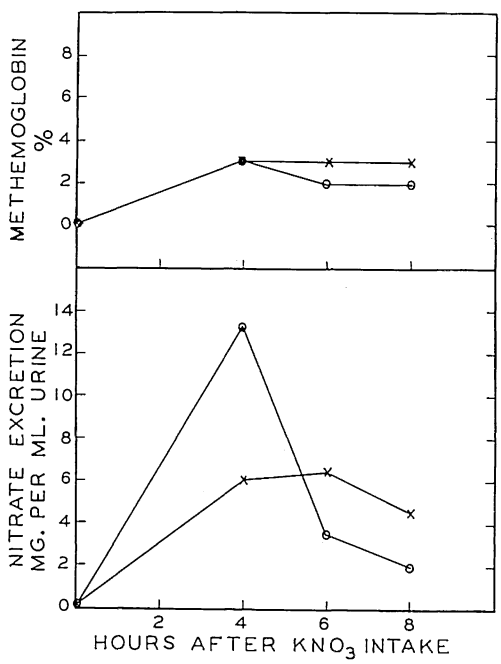


FIG. 2. Urinary nitrate excretion and methemoglobin formation in two calves receiving 25 g. KNO_3/100 lb. body weight.

eight-day experiment. The latter calf showed maximum hemoglobin-to-methemoglobin conversion values of between 35 and 50% on four of the eight days.

The toxic symptoms observed throughout this study, for both the single and multiple KNO_3 doses, were relatively mild, as compared to nitrate poisoning symptoms generally seen both in dairy and in beef cattle under practical farm conditions, at much lower levels of nitrate intake. This immediately poses a question as to the true nature of so-called nitrate poisoning observed on the farm. The apparent discrepancy between symptoms induced experimentally and those observed under practical conditions may be owing either entirely or in part to: *(1)* Form of nitrate ingested (inorganic vs. organic); *(2)* diet, rumen function, and nutritional status of animals in conjunction with amount of environmental stress; *(3)* age and factors associated with stress of milk and meat production, and *(4)* level of nitrate intake, particularly as related to total period of intake and amount consumed at any given time.

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COMPARATIVE METABOLISM OF CALCIUM AND STRONTIUM IN LACTATION

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SUMMARY

The comparative metabolism of calcium and strontium in the lactating and nonlactating goat was studied, using double tracer methods. At steady state, calcium was preferentially secreted into milk by a factor of about 11 over strontium in its passage from diet to milk. With corollary data from nonlactating animals, it was found that the largest amount of discrimination took place in the gastrointestinal absorption of these alkaline earths, calcium being absorbed from three to four times that of strontium. The over-all discrimination of 0.09 between milk and diet resulted primarily from the following discrimination factors: absorption, 0.24; urinary excretion, 0.60; transfer from plasma to milk, 0.61. Under steady state conditions, the relative Sr^*/Ca^* ratios were diet = 1, plasma = 0.23, skeleton = 0.23, milk = 0.09, and urine = 4.0. These values indicate that there is no differential movement of calcium and strontium between blood and bone, and that strontium is preferentially excreted in the urinary process.

Radiostrontium is at present detectable by low-level counting techniques in the food chain (soil, plants, milk, animals, and man); this radioactivity originates from nuclear detonation *(8)*. The progression of radiostrontium along the food chain generally follows, and is related to calcium movement *(3, 12)*. The amount of radiostrontium in the human population for a given amount of fallout will depend on many factors, especially the differential movement of calcium and strontium in the food chain and the strontium^{90} content of the dietary calcium. In the United States, it has been estimated that from 70 to 80% of the calcium in the human dietary originates from milk or milk products. Fortunately, considerable discrimination against strontium in favor of calcium occurs in the passage of these alkaline earths from the diet of the dairy animal to the milk. Were it not for this differential metabolism, the Sr^{90} content of the human population would be about five times higher than otherwise.

It has been shown that the Sr^{90}-to-Ca ratio of milk will be from 0.09 to 0.16 that of the ration being consumed by the animal. This was determined by experimental studies using radioisotopes of strontium and calcium *(4)*, analysis of feed and milk for fallout Sr^{90} and stable calcium *(1)*, and analysis of feed and milk for stable calcium and stable strontium *(6)*. The present paper presents confirmation of the over-all discrimination in the goat; in addition, more detailed studies are described that give information on the comparative metabolism of these two elements in a ruminant, with emphasis on the physiological mechanisms responsible for their differential behavior.

Received for publication February 7, 1958.

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MATERIALS AND METHODS

The terminology used in discussing the comparative movement and differential handling of calcium and strontium is that proposed by Comar *et al. (5)*. The "Strontium-Calcium Observed Ratio" is defined as the ratio of strontium to calcium in any product compartment, divided by the ratio in a precursor compartment, as follows:

$$OR_{\text{sample-precursor}} = \frac{\text{Sr/Ca of sample}}{\text{Sr/Ca of precursor}} \quad (1)$$

The concentrations of Ca^* and Sr^* in tissues or excretions are expressed as per cent of administered dose.

Lactating goats—daily isotope ingestion. Two lactating goats, each of about 36 kg. body weight, were placed in metabolism stalls, as described by Comar *(2)*, for the quantitative collection of feces and urine. A good-quality mixed hay was fed with a commercial dairy feed containing 16% crude protein. After a preliminary conditioning period of about a week, the simultaneous oral administration of Ca^{45} and Sr^{89} by capsule was begun. Each goat received 200 μc. Ca^{45} and 66 μc. Sr^{89} twice daily by capsule, at 8 A.M. and 4 P.M. for 12 days;

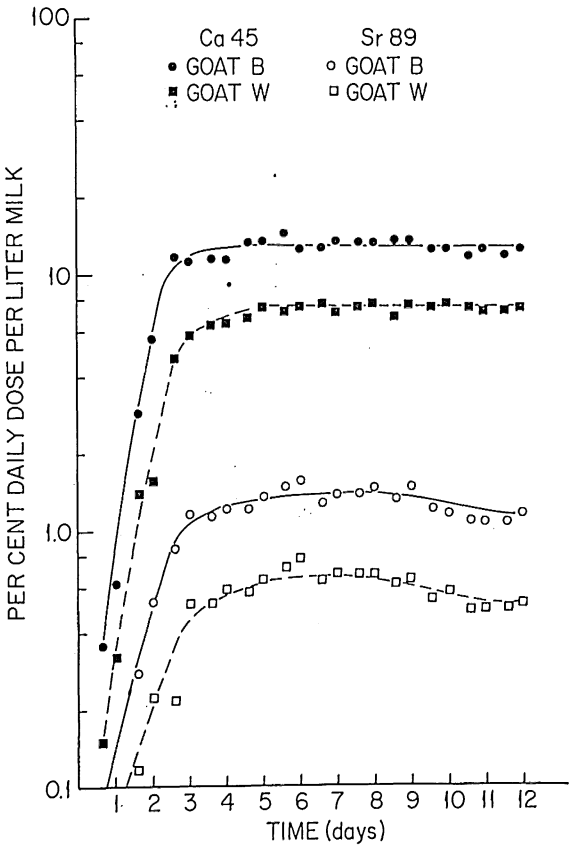


FIG. 1. Levels of daily ingested Ca^{45} and Sr^{89} appearing in the milk of goats.

the isotope preparations were essentially carrier-free and in the form of the chloride salt. The feces and urine were collected only once daily, and the animals were milked twice daily. On the 13th day, the goats were killed and tissue samples were obtained. The milk, tissues, and excretions were analyzed for Ca^{45} and Sr^{89} by the differential absorption procedure *(2)* and results expressed as "per cent of the daily ingested dose."

Nonlactating goats—single-dose studies. As will be pointed out later, it was necessary to compare results after oral and intravenous administration, to obtain information on the role of gastrointestinal absorption in causing discrimination. Single oral doses of Ca^{45} and Sr^{89} were given by gavage to two goats; heparinized blood samples were taken from the jugular vein at periodic intervals over a three-day period. Two other nonlactating goats of comparable body size were given 600 μc. Ca^{45} and 60 μc. Sr^{85} intravenously. Frequent blood samples also were obtained from these animals through an indwelling catheter placed in the jugular vein. The time intervals of blood sampling from both methods of administration were as shown (Fig. 2). The plasma of the "oral" group was assayed for the two beta-emitting isotopes, Ca^{45} and Sr^{89}, by the differential absorption method. In the intravenous group, Ca^{45}, a pure beta emitter, was determined by conventional beta-counting methods with a Geiger-

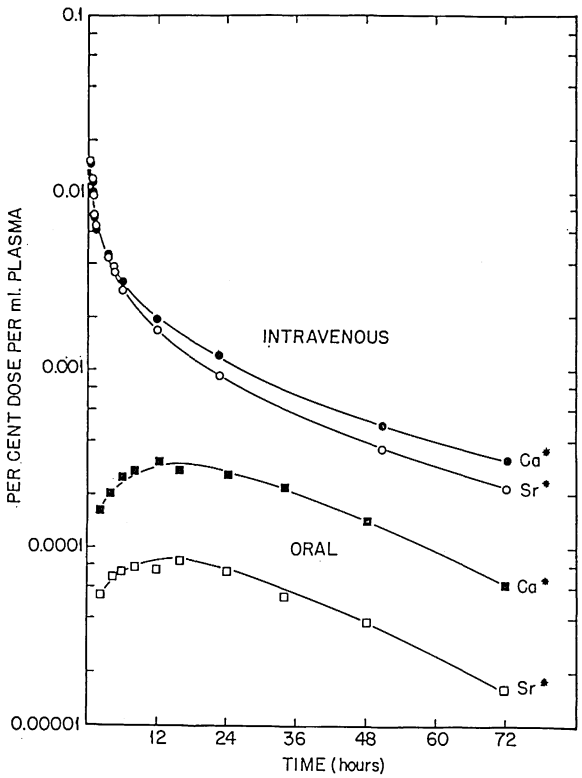


FIG. 2. Levels of Ca* and Sr* in the plasma of goats after a single oral or a single intravenous dose of the radioisotopes.

Muller detector and Sr^{85}, a pure gamma emitter, by liquid counting in a well-type scintillation detector. At the levels of activity used, the beta count did not contribute to the gamma count, and vice versa. At five days after dosage, the intravenously injected goats were killed and various bone samples were selected for radioassay.

RESULTS AND DISCUSSION

Lactation. As shown (Figure 1), the Ca^{45} and Sr^{89} content of the milk, expressed as per cent of the daily dose per liter, increased rapidly until a plateau level was attained at about three days. After this time, no appreciable change in the milk levels occurred. A slight decrease in the Sr^{89} levels between the 9th and the 12th day was observed; if real, no physiological explanation can be offered. It should be pointed out also that the Ca^{45} and Sr^{89} concentrations in the plasma did not change after three to four days of daily radioisotope administration, and that the daily excretion of these isotopes in the urine and feces likewise remained essentially the same. Thus, these animals may be considered at "steady state", in that the radioisotope concentrations in the body fluids and excretions had attained some constant function of the intake. Under these conditions, the experimental animals were secreting about 10% of the Ca^{45} and about 1% of the Sr^{89} ingested per day per liter of milk (Figure 1 and Table 1).

TABLE 1

Summary of levels of Ca^{45} and Sr^{89} in plasma and milk and observed ratios in the lactating goat at steady state

	Goat B	Goat W
Ca^{45} in milk (% of daily dose/liter)	12.8	7.3
Sr^{89} in milk (% of daily dose/liter)	1.3	0.61
Ca^{45} in plasma (% of daily dose/liter)	0.87	0.58
Sr^{89} in plasma (% of daily dose/liter)	0.24	0.12
$OR_{milk\text{-}diet}$[a]	0.10	0.083
$OR_{plasma\text{-}diet}$	0.27	0.20
$OR_{milk\text{-}plasma}$	0.38	0.41

[a] $OR_{sample\text{-}precursor} = \dfrac{Sr^*/Ca^* \text{ in sample}}{Sr^*/Ca^* \text{ in precursor}}$.

Under steady-state conditions, a comparison of the various OR values (calculated from Equation 1) indicates the degree of discrimination occurring in the movement of Ca^{45} and Sr^{89} among compartments (diet, plasma, and milk). These have been summarized (Table 1). Of considerable importance is the overall differential movement of calcium and strontium from diet to milk. The $OR_{milk\text{-}diet}$ was found to be 0.10 and 0.08 for Goat B and Goat W, respectively, indicating that dietary calcium was preferentially secreted into milk by a factor of ten to 12 times that of dietary strontium. These values are in agreement with the $OR_{milk\text{-}diet}$ values previously reported for the bovine, which averaged about 0.13. In the movement from plasma to milk, calcium was preferentially secreted by a factor of 2.5 over strontium as shown by the $OR_{milk\text{-}plasma}$ values of 0.38 and 0.41. In the cow, the $OR_{milk\text{-}diet}$ was found to be 0.14, the $OR_{plasma\text{-}diet}$, 0.37, and the $OR_{milk\text{-}plasma}$, 0.38 *(3)*; from these data, it is apparent that the

differential movement of calcium and strontium from plasma to milk in the cow was identical with that in the goat.

From a knowledge of the specific activities of Ca^{45} in the diet and the milk of the goats, it was possible to estimate the comparative contribution of dietary calcium and skeletal calcium to milk calcium by applying the usual principles of isotope dilution. The specific activities of the diet and milk averaged 8.0 and 6.8% of the daily ingested dose of Ca^{45} per gram of total calcium, respectively; from these values, it was calculated that approximately 85% of the milk calcium originated from the diet and 15% primarily from the maternal skeleton. Thus, milk calcium is comprised essentially of dietary calcium; this same relationship would hold for radiostrontium, since there are negligible differences in the movement of calcium and strontium between blood and bone *(5, 10, 14, 16)*. From these considerations, it becomes evident that, after a few days of daily ingestion, the milk levels of Sr^{90} represent mainly the activity that was ingested over the previous few days and would constitute an immediate and contemporary indication of the fallout Sr^{90} available to the lactating animal. This is also borne out by the fluctuations observed in the fallout Sr^{90}-to-calcium ratios in fresh milk samples taken from the Chicago dairies; these changes were correlated with nuclear tests and their relation to seasonal variations in feeding practices *(15)*.

Absorption and excretion. The levels of Ca^{45} and Sr^{89} in per cent of daily dose per day, in the milk, excretions, and body of the goat at steady state, are summarized (Table 2). The fecal excretions of both calcium and strontium were high, as judged from direct measurement—87.5 and 101% of the ingested dose,

TABLE 2

Levels of Ca^{45} and Sr^{89} in milk, excretions, and body of the goat at steady state (in per cent of daily dose per day) and observed ratios

	Goat B	Goat W	Mean
Milk			
Ca^{45}	14.0	6.8	10.4
Sr^{89}	1.4	0.57	1.0
Feces (direct measurement)			
Ca^{45}	85	90	87.5
Sr^{89}	94	108	101
Feces (by difference[a])			
Ca^{45}	77	82	79
Sr^{89}	94	95	95
$OR_{feces\text{-}diet}$[b]	1.13	1.12	1.12
Urine			
Ca^{45}	0.42	0.74	0.58
Sr^{89}	2.3	1.8	2.1
$OR_{urine\text{-}diet}$	5.5	2.5	4.0
$OR_{urine\text{-}plasma}$	20.5	12.1	16.3
In body (estimated from bone analysis)			
Ca^{45}	9.1	11	9.8
Sr^{89}	2.1	2.2	2.2

[a] Intake—(estimated amount in body + urinary excretion + milk secretion).

[b] $OR_{sample\text{-}precursor} = \dfrac{Sr^*/Ca^* \text{ in sample}}{Sr^*/Ca^* \text{ in precursor}}$.

respectively. Because of the unrealistic value of 108% obtained for Goat W, estimates were made by calculating the amounts retained in the body from bone analyses and subtracting the sum of this value, the urinary excretion, and the milk secretion from the intake. These values (feces, by differences) appeared to be quite reasonable. Because of the high fecal excretions and the inherent inaccuracies, an alternate experiment was done to give estimates of differential absorption. In this study, a comparison was made of plasma levels of radiostrontium and radiocalcium, after oral and intravenous administration to nonlactating goats. The data are given (Figure 2).

Comparison of the plasma levels (Figure 2) shows clearly that there was little difference between radiocalcium and radiostrontium concentrations following injection; after oral administration, however, the plasma levels of radiocalcium were significantly higher than those of radiostrontium. It is apparent, then, that radiostrontium was being absorbed less effectively than was radiocalcium. The extent of the absorptive discrimination was estimated by comparing the $OR_{plasma\text{-}dose}$ values after both modes of administration. The $OR_{plasma\text{-}dose}$ after intravenous injection was 1.0 for the first 6 hr.; thereafter, it decreased until a value of 0.7 was attained at 72 hr. In the goats receiving the radioisotopes by the oral route, the $OR_{plasma\text{-}dose}$ was about 0.3 when absorption was maximum at about 12 hr., and decreased to a value of about 0.27 at 72 hr. Thus, it is inferred that the nonlactating goat was able preferentially to absorb radiocalcium over radiostrontium by a factor of about three, and that this also would be true for the lactating animal.

The preferential absorption of radiocalcium over radiostrontium by the goat is contrary to reports of equal absorption of these cations in the cow *(11)*. However, the data in the cow were obtained from single-dosage studies of one isotope at a time to the same animal on different occasions or to different animals. Therefore, they are not so precise as double-tracer studies. Further investigations are needed to answer the question of gastrointestinal discrimination in the bovine.

In the urinary process (as shown in Table 2), strontium was preferentially excreted as shown by the $OR_{urine\text{-}diet}$ values of 5.5 and 2.5; the comparative plasma clearance by the kidney was 12 to 20 times greater for strontium than for calcium. These comparative clearance values differ from those of three to five found in man and rats, but agree with the clearance figure of about 24 obtained in cattle *(4)*.

Tissue analysis. The strontium-calcium observed ratios, and the Ca^{45} and Sr^{89} content of various tissues from the lactating animals, are shown (Table 3). First, it may be noted that the $OR_{tissue\text{-}diet}$ values for the bone samples were relatively uniform, although considerable variation was found in the per cent of the daily ingested activities present. In Goat B, for example, the Ca^{45} and Sr^{89} contents of the jawbone were almost three times those of the femur shaft; yet, the $OR_{tissue\text{-}diet}$ values for both bones were almost identical. The teeth, on the other hand, had an observed ratio higher than that of the other bony tissues, probably caused by the exchange of Ca^{45} and Sr^{89} directly into the tooth enamel

TABLE 3

Levels of Ca^{45} and Sr^{89} and observed ratios (OR) in various tissues of the lactating goat at steady state

Tissue	Ca^{45} (% daily dose per g. tissue)	Sr^{89} (% daily dose per g. tissue)	$OR_{tissue-diet}$[a]
	Goat B		
Femur shaft	0.014	0.0035	0.26
Femur epiphysis	0.018	0.0035	0.20
Rib shaft	0.017	0.0045	0.26
Rib epiphysis	0.033	0.0070	0.21
Jaw (angle)	0.037	0.0092	0.25
Incisor tooth	0.010	0.0037	0.36
Plasma	0.00087[b]	0.00024[b]	0.27
	Goat W		
Femur shaft	0.022	0.0045	0.20
Femur epiphysis	0.021	0.0041	0.20
Rib shaft	0.025	0.0057	0.22
Rib epiphysis	0.025	0.0054	0.22
Jaw (angle)	0.055	0.0107	0.20
Incisor tooth	0.0056	0.0018	0.32
Plasma	0.00058[b]	0.00012[b]	0.20

[a] $OR_{tissue-diet} = \dfrac{\text{Sr/Ca tissue}}{\text{Sr/Ca diet}}$.

[b] Plasma values were given as % daily dose per ml.

from the bolus during rumination; the $OR_{bolus-diet}$ should be close to unity. It should also be noted that the $OR_{plasma-diet}$ and $OR_{skeleton-diet}$ values were quite similar in both animals; the average $OR_{skeleton-plasma}$ value of 0.95 (Table 2) demonstrates that no appreciable discrimination occurred in the blood-bone system. This has been previously shown in rats *(5, 16)*, rabbits *(14)*, and embryonic-chick bone cultures *(10)*.

The Ca^{45} and Sr^{85} analyses of bone samples from the singly-injected, nonlactating goats are presented (Table 4). These data again demonstrate the variability with which different types of bones accumulate radiocalcium and

TABLE 4

Levels of Ca^{45} and Sr^{85} and observed ratios (OR) in the skeleton of the nonlactating goat after intravenous administration

Tissue	Ca^{45} (% dose/g fresh wt.)	Sr^{85} (% dose/g fresh wt.)	$OR_{tissue-dose}$[a]
	Goat 1		
Tibia epiphysis	0.0071	0.0055	0.77
Tibia shaft	0.0056	0.0041	0.73
Rib epiphysis	0.020	0.039	0.52
Rib shaft	0.014	0.021	0.64
	Goat 2		
Tibia epiphysis	0.0078	0.0064	0.83
Tibia shaft	0.0090	0.0074	0.83
Rib epiphysis	0.044	0.033	0.76
Rib shaft	0.027	0.019	0.71
Incisor tooth	0.0045	0.0038	0.83

[a] $OR_{tissue-dose} = \dfrac{\text{Sr/Ca of tissue}}{\text{Sr/Ca of dose}}$.

radiostrontium; also, the similarity in the strontium-calcium observed ratios. This can be illustrated by reference to the amounts of activity present in the tibia shaft and rib shaft of Goat 1; the latter tissue accumulated about four times as much radiocalcium and radiostrontium as did the tibia shaft; the $OR_{tissue\text{-}dose}$ values were about the same for both types of bone. The lower $OR_{tissue\text{-}dose}$ values for the ribs in contrast to the tibias are probably real, and reflect the greater ability of the ribs to equilibrate with plasma as the plasma changes with time. After the intravenous dose, the $OR_{tissue\text{-}dose}$ for the incisor tooth of Goat 2 was identical with that of the tibia; thus, the tooth was unable to metabolize these cations differently than certain parts of the skeleton; therefore, this supports the idea that the higher $OR_{tissue\text{-}dose}$ of the tooth after oral administration was related to surface effects.

From the rather constant $OR_{bone\text{-}diet}$ values found in the continuously fed and singly injected goats, it may be implied that, in the long run, the concentration of fallout strontium to total calcium will be identical in all sites of the skeleton. Therefore, "hot spots" in different types of bone or different areas of any bone, as demonstrable with single injections of Ca^{45}, Sr^{90}, or radium *(7, 13)*, will not be formed, at least on a macro-scale. Fallout strontium, being ingested at fairly constant levels over the years, will be uniformly dispersed throughout the bone calcium accreted during that period. Kulp *et al. (8)* have calculated the total skeletal load of Sr^{90} from the analysis of a single type of bone and a correction factor based on the variability with which different types of bone accumulate radiostrontium after a single intravenous injection. This may be applicable to older individuals exposed to fallout Sr^{90} over a relatively short segment of their skeletal history. This correction will be less valid as the length of exposure to Sr^{90} increases, and certainly would not apply to young persons, whose entire skeleton was formed since the time when the biosphere was initially contaminated with Sr^{90}. These considerations have been treated recently by Langham and Anderson *(9)*.

Over-all discrimination. To quantitate the contribution of individual physiological processes to the over-all discrimination, the term strontium-calcium discrimination factor (DF) has been defined as follows *(5)*:

$$OR = (DF_{absorptive})\ (DF_{urinary})\ (DF_{lactational})\ \ldots\ (DF_{n}) \tag{2}$$

In the lactating animal, it appears that the only processes significantly operative in causing discrimination are absorption, urinary excretion, and secretion into milk. The following equations have been developed, similar to those for discrimination in the pregnant animal *(17)*:

$$DF_{absorptive} = \frac{100 - F_{Sr}}{100 - F_{Ca}} \tag{3}$$

$$DF_{urinary} = \frac{(100 - F_{Ca}) - \left(U_{Sr} \times \dfrac{100 - F_{Ca}}{100 - F_{Sr}}\right)}{B_{Ca} + L_{Ca}} \tag{4}$$

$$DF_{lactational\text{-}body} = \left(\frac{B_{Sr}}{B_{Ca}}\right)\left(\frac{B_{Ca} + L_{Ca}}{B_{Sr} + L_{Sr}}\right) \tag{5}$$

$$DF_{lactational\text{-}milk} = \left(\frac{L_{Sr}}{L_{Ca}}\right)\left(\frac{B_{Ca} + L_{Ca}}{B_{Sr} + L_{Sr}}\right) \tag{6}$$

where

F_{Sr} and F_{Ca} = per cent of ingested dose of Sr^{89} and Ca^{45}, respectively, in feces
B_{Sr} and B_{Ca} = per cent of ingested dose of Sr^{89} and Ca^{45}, respectively, in the body
L_{Sr} and L_{Ca} = per cent of ingested dose of Sr^{89} and Ca^{45}, respectively, in the milk
U_{Sr} and U_{Ca} = per cent of ingested dose of Sr^{89} and Ca^{45}, respectively, excreted in the urine

In derivation, the assumptions are made that the urinary excretion of each element is proportional to the amount absorbed, and that the amount secreted into the milk is proportional to the amount absorbed, minus that excreted into the urine.

Using the mean values (from Table 2), the following are calculated:

$$DF_{absorptive} = 0.24$$
$$DF_{urinary} = 0.60$$
$$DF_{lactational\text{-}milk} = 0.61$$
$$DF_{lactational\text{-}body} = 1.4$$

These DF values then permit a comparison of the importance of the different physiological processes in bringing about the discrimination between calcium and strontium. It is clear that the preferential absorption of calcium from the gastrointestinal tract is of major significance. Even though the total urinary excretion of calcium in the goat is small, this process contributes to the discrimination in an important way; this is because of the much greater plasma clearance of strontium, and because of the relatively small amounts of the two elements that are absorbed and metabolized by the body. The lactation process is of equal importance and tends to reduce the strontium-calcium ratio in the milk; whereas, it raises the strontium-calcium ratio in the body.

ACKNOWLEDGMENTS

The authors wish to express their gratitude for the capable assistance of Dr. I. Kanno, Visiting Scientist from Sendai, Japan, and Mr. Charles Phipps. The assistance of the staff of the animal laboratory, in the care of the experimental animals, is gratefully acknowledged.

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EFFECT OF HEAVY CONCENTRATE FEEDING BEFORE CALVING UPON LACTATION AND MAMMARY GLAND EDEMA [1]

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SUMMARY

Heavy grain-feeding before calving resulted in no increase in the yield of fat-corrected milk during the first 84 days of lactation. Nine cows and nine heifers received, in addition to a basal roughage ration, a supplement of 378 and 273 lb. grain, respectively, during a 6-wk. prepartum period. A similar number of controls received no grain. During lactation all animals were fed roughage and grain according to maintenance and production requirements. Supplemented animals made significantly greater ($P = < 0.01$) increases in live weight but evidenced no significant increase in the incidence or severity of mammary edema as determined by visual ratings and linear measurements. Heifers showed more edema at calving than did older cows.

Thirty years ago, Boutflour *(2)* introduced a method of preparing a cow for lactation which he called "steaming up." In this procedure the cow receives concentrates 6 wk. before the expected date of calving and the allotment is increased weekly. In the final week of the dry period, the amount of grain is increased to 75% of the estimated grain requirement of early lactation.

The stated objects of steaming up are to build up the cow's body reserves and to accustom her to the heavy grain ration she should receive in early lactation. Boutflour *(3)* claimed that milk production in the subsequent lactation is increased by this technique. Although steaming up is a fairly common practice in Britain, it has received little attention from research workers in Britain and no attention from those in the United States. Blaxter *(1)* obtained significantly greater milk yields in early lactation when cows were steamed up for a 6 wk. period by feeding up to 6 lb. of concentrates daily above a control ration consisting entirely of bulky feeds. A third group of cows received supplemental bulky feeds in amounts equivalent in net energy content to the grain fed to the steamed-up group. The milk yields of the third group were increased over the controls, but not significantly.

The effect of a high plane of nutrition compared with a low plane during the dry period also has been studied by Popoff *(9)* and Skrodels *(10)*. These workers obtained increases of up to 70% for the complete lactation as a result of supplementation of the basic dry-period ration with concentrates.

A second aspect of this study arose as a result of the common belief among dairymen that grain feeding during the dry period increases the incidence and severity of mammary edema at calving. However, there is little experimental

Received for publication September 13, 1957.

[1] Data presented in this paper are taken from a thesis submitted by J. F. D. Greenhalgh in partial fulfillment of the requirements for the M.S. degree.

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evidence bearing on the question. Wylie and Hinton *(13)* observed that switching from a plan of normal grain feeding in the dry period to heavy grain feeding in late lactation and no grain in the dry period reduced udder edema and increased production. However, Fountaine, Parrish, and Atkeson *(5)*, in a more thorough investigation, found that the amount of edema at calving was not related to grain feeding during the dry period.

The principal purpose of the present experiment was to examine the effect of steaming up upon the production of cows kept under typical American conditions of feeding and management. Further evidence concerning the effect of such concentrate feeding upon udder condition appeared desirable, in view of the scarcity of studies on the subject and the disagreement in results described above. The problem of measuring accurately the degree of mammary congestion also appeared to be of interest, since no objective method of evaluation of the condition was available.

EXPERIMENTAL PROCEDURE

Animals. Thirty-six cows and heifers of the University dairy herd were employed in the experiment. The five major dairy breeds were represented, but Holsteins predominated. Eighteen cows were placed in two groups of nine with animals of the same breed being equally divided between groups, and 18 heifers were similarly apportioned. Apart from division by breed, the assignment of animals to Group I, Supplemented, or Group II, Control, was at random. All animals were due to calve between October, 1953, and February, 1954.

Feeding and management. Throughout the experiment, all animals were housed in stanchion barns and during lactation were milked by machine at 4:15 A.M. and 3:45 P.M. All feeds were weighed out daily and refusals weighed back. The feeds consisted of good-quality corn silage, medium-quality alfalfa hay, and a 15.4% total protein grain mixture composed of yellow shelled corn, 40, oats, 35, wheat bran, 10, soybean meal, 5, linseed meal, 7.5, steamed bone meal, 1, and trace-mineralized salt 1.5%.

The experimental feeding period began 42 days before each animal was expected to calve. For the whole experiment all animals received daily 2 lb. of silage and 1-½ lb. of hay per 100 lb. live weight. Before calving, grain was fed to the supplemented group only. During lactation all animals were fed according to Morrison's *(8)* optimum standards. Grain was fed in amount sufficient to meet the difference between the TDN provided by the hay and silage, and the calculated requirement. The prepartum and postpartum concentrate feeding schedules are shown (Table 1).

Observations recorded. Milk records were kept for the first 12 wk. of lactation, beginning the fourth morning after calving. Fat tests, according to the Babcock method, were made on milk samples taken from Monday through Thursday each week.

All animals were weighed 42 and seven days before the predicted calving date, and seven and 84 days after calving. Each weight is the mean of three days' weighings carried out at 1 P.M. each day.

TABLE 1
Concentrate feeding schedule

Days prepartum[a]	Daily grain to supplemented group[b]		Days postpartum	Daily grain after calving[c]	
	Cows	Heifers		Cows	Heifers
	(lb.)			*(lb.)*	
42–36	5	4	0–4	Discretion of herdsman	
35–29	7	5	5–8	6	4
28–22	9	6	9–12	8	6
21–15	10	7	13–16	10	8
14– 8	11	8	17–88	According to TDN requirement	
7–parturition	12	9			
Av. grain per animal	382	260			

[a] Schedule based on expected calving date.
[b] Control group received no concentrates during dry period.
[c] Grain fed at same rate to supplemented and control animals after calving.

In any study of udder edema, there is the fundamental problem of measuring the amount of swelling and inflammation present. Five methods of measurement were tested by Wilson *(12)*, but in this experiment these were limited to two. In the first method, the severity of the edema was estimated independently by two experienced judges, who examined each animal three days after calving. They rated the udder according to a simple scale, in which 1 represented slight edema and 5 severe edema.

A second method of physical measurement was employed, the principle being to compare the size of the udder at calving with its normal size. Thus, the udder was first measured three days after calving, and further measurements were made 30 days after calving, when a normal condition could be presumed. The measurements, which were made with a tape, were as follows: *a)* udder length: the distance from a mark made on the escutcheon, along the sole of the udder to the junction of the udder with the abdominal wall; *b)* udder width: the distance from the junction of the udder with the abdomen on one side to the same point on the other side, the tape being placed midway between the front and rear teats.

These measurements were made at 8:30 A.M., 4 hr. after milking. Some difficulty was experienced in finding a suitable agent for making a mark on the escutcheon, and the substance eventually employed was red fingernail-varnish.

Statistical analysis. In the original design of the experiment it was planned that there should be two treatment groups, each containing two subclasses; that is, a supplemented group divided into subclasses of nine cows or nine heifers, and a control group similarly divided. However, the failure of one cow to calve, and the loss of live weight data resulting from early calving, led to the formation of disproportionate subclass numbers. Consequently, the necessary analyses of variance were made by the method of fitting constants *(6)*.

RESULTS

The average length of the prepartum feeding period was 43 days for cows and 40 days for heifers. Feed refusals throughout the trial were negligible. Milk yields, live weight changes, and mammary edema measurements recorded during the experiment are shown (Table 2). In this table, direct comparisons between

TABLE 2

Milk yields, live weight changes, and mammary edema measurements

	Measurement	Means				Residual standard deviation	Differences	
		Supplemented		Control				
		Cows	Heifers	Cows	Heifers		Supplemented vs. control	Cows vs. heifers
I	Milk yields							
	Daily yield of FCM in first 84 days of lactation *(lb.)*	47.5 (9)[a]	39.4 (9)	49.4 (8)	38.3 (9)	± 8.1	− 0.1	9.6[b]
	Daily yield of actual milk in first 84 days of lactation *(lb.)*	46.8 (9)	41.3 (9)	49.5 (8)	40.4 (9)	± 9.7	− 0.7	7.3[c]
II	Live weight changes							
	Between 42 and seven days prepartum *(lb.)*	+67.8 (9)	+80.4 (7)	+43.0 (4)	+42.7 (7)	±29	30.5[b]	− 1.4
	Between seven and 84 days postpartum *(lb.)*	−43.9 (9)	−17.2 (9)	−24.3 (8)	+ 2.0 (9)	±41	20.1	27.1
III	Mammary edema measurements							
	Visual rating (1 = slight, 5 = severe edema)	1.86 (8)	3.61 (9)	2.35 (7)	3.09 (9)	± 0.84	− 0.02	− 1.26[b]
	Udder length measurement. Reduction in length from three to 30 days postpartum *(in.)*	2.58 (6)	4.14 (7)	1.79 (7)	3.44 (9)	± 2.02	0.70	− 1.60[c]
	Udder width measurement. Reduction in width from three to 30 days postpartum *(in.)*	2.42 (6)	3.21 (7)	0.93 (7)	2.33 (9)	± 2.55	1.13	− 1.10

[a] Figures in parentheses are numbers of animals.
[b] Significant at the 1% level of probability.
[c] Significant at the 5% level of probability.

subclass means are permissible, since all interactions were nonsignificant. The subclass standard deviations were tested for homogeneity and were found to be combinable.

As one would expect, the FCM production of mature cows was significantly greater ($P = <0.01$) than that of first-calf heifers. The differences between the supplemented and the control groups in their production of both actual milk and FCM during the first 84 days of lactation were nonsignificant. Production curves drawn by plotting yield against time did not suggest that differences between groups would develop later in the lactation.

As shown (Part II of Table 2), the mean live weight gain for nine supplemented cows from 42 to seven days prepartum was 67.8 lb., and for four control cows, 43.0 lb. Gains for seven supplemented heifers averaged 80.4 lb., compared with 42.7 lb. for seven controls. Group I animals made significantly greater weight gains ($P = <0.01$) during the dry period. After calving the intra-class variability was great and mean differences were nonsignificant.

Results of the edema study appear in Part III of Table 2. Assessment of the severity of the condition at calving by means of judges' ratings showed a highly significant difference between cows and heifers, but no difference as a response to treatment. Measurements of the decrease in udder length between three and 30 days postpartum again indicated differences between age groups, but at a lower level of significance. The measurements of the reduction in udder width showed no significant differences between ages or treatments.

The agreement between methods of measuring edema was tested by calculating a number of simple and multiple correlation coefficients, which are shown (Table 3).

TABLE 3

Comparison of the different methods of measuring udder edema

Correlation between:	"r" value
1. The ratings of one judge and those of the other, $r_{x_I \cdot x_{II}}$	0.8201[a]
2. Ratings and udder length decreases, $r_{x \cdot y}$	0.5123[a]
3. Ratings and udder width decreases, $r_{x \cdot z}$	0.1654
4. Ratings and both measurements, $r_{x \cdot yz}$	0.5138[b]
5. Udder length decreases and udder width decreases, $r_{y \cdot z}$	0.3919[b]

[a] Significant at 1% level of probability.
[b] Significant at 5% level of probability.

This table shows that the judges agreed well when making their independent estimates of the amount of edema present. When the measurements were compared with the ratings, the decrease in udder length between three and 30 days postpartum appeared to evaluate the condition much better than the decrease in udder width in the same period.

DISCUSSION

Milk yields and live weight changes. The feeding of a supplementary grain ration prior to calving could, conceivably, bring about a subsequent increase in milk production in a number of ways. The first and most obvious mode of

action is that deposits of body fat laid down in the dry period could be "milked off" during lactation. Secondly, there is the possibility that an improved supply of nutrients in late pregnancy could encourage extra development of milk secretory tissue; hence, an augmented milk flow. Finally, steaming up could improve milk production by what might be called a metabolic preparatory effect. It is generally considered advisable to increase postpartum feed intake gradually, to avoid trouble from digestive disorders and, possibly, milk fever, with the result that nutrient intake may fall behind nutrient requirements. Consequently, at a time when milk production should be stimulated, a brake is placed on secretion. However, as a result of accustoming the cow to heavy grain feeding before calving, the build-up of nutrient intake could be accelerated, and the cow could be brought up to full feed soon after calving.

Results of this experiment provide no evidence that any of these modes of action was operating. Although supplemented animals made significantly greater weight gains before calving and appeared to lose this weight advantage after calving, their milk yields were not increased over the controls. It is possible that the method of feeding after calving suppressed any effects of steaming up. All animals may have been fed so liberally that the supplemented animals had no opportunity to make efficient use of their extra weight increases. This is a matter for conjecture, but it is suggested that the conversion of feed to milk via the intermediate step of body fat is likely to be an unprofitable procedure. The third suggested mode of action, the metabolic preparatory effect, may not have had a chance to manifest itself. The experimental animals were not brought on to full feed immediately after calving, but the build-up was faster than is normally the case (see Table 1).

Before turning to other experiments in which a high plane of nutrition during the dry period has proved its worth, it must be stressed that steaming up, as promoted by Boutflour *(3)*, involves the supplementing with grain of normally adequate rations. Consequently, when measuring the effect of steaming up, it is important that control animals should be fed at such a level that they can make live weight increases commensurate with the growth of the fetus and accompanying tissues, i.e., controls must not actually lose flesh during the dry period. In New Zealand *(4, 7)*, high and low planes of nutrition during the dry period were compared by their effect on the following lactation. In these experiments, the control animals were made to lose weight prior to calving, and so the milk-yield differences represent a response to adequate nutrition compared with underfeeding. Steaming up, on the other hand, is overnutrition and its true value can be assessed only by comparing it with adequate nutrition.

In the investigation of steaming up conducted by Blaxter *(1)*, cows whose rations were supplemented either with grain or with bulky foods gained approximately 80 lb. per head during the period 42 to seven days prepartum. Controls gained only 13 lb. per head, an amount which represents an actual loss of flesh when the increase in weight of the fetus and its membranes is taken into account *(11)*. Consequently, steaming up was being compared with underfeeding. In the experiment reported here, however, supplemented animals gained an

average of 73.3 lb. and controls 42.8 lb., from 42 to seven days prepartum. The latter weight increase indicates that the controls were being fed an adequate ration.

The present experiment demonstrates the effect of steaming up on milk production, in an area in which cows are usually well-fed during the dry period.

Mammary edema. The first aim in measuring the udder edema encountered in this experiment was to compare objective and subjective methods, with a view to establishing a satisfactory objective method of evaluating the condition. The second aim was to investigate any connection between prepartum grain feeding and mammary edema.

It is logical to assume that an over-all estimate of the degree of swelling in an irregularly shaped body should be more accurate than any one linear measurement; this assumption is confirmed by the results of this experiment. Judgment by eye appeared to be a more satisfactory method of assessing the severity of the condition than measurements of udder length and width. Thus, Table 3 shows that the correlation between the ratings of one judge and those of the other was far closer than any of the other correlations investigated. This conclusion does not solve the problem of finding an objective measurement, but it does suggest that for any one experiment judgment by the practiced eye is a fairly satisfactory method of measuring edema.

The length measurement of the udder appeared to be more useful than the width measurement in evaluating the edema. The apparent reason for this is that length measurements included the accumulation of fluid often seen at the fore end of the udder, whereas width measurements did not. When measurement changes from three to 30 days postpartum were expressed on a percentage basis, and differences in basic udder size between cows were eliminated, the correlation between the measurements and judges' ratings was not increased.

The symptoms of mammary edema are well known, but their cause is still a matter for speculation. Although the relation between prepartum grain feeding and an accumulation of lymph in the udder is not obvious, dairymen often believe that such a connection exists. However, in this experiment, feeding as much as 9 or 12 lb. of grain per day before calving did not increase the amount of edema. Singificant differences occurred between cows and heifers, but there were no interaction effects, i.e., there was no evidence that grain feeding increased edema in cows but not in heifers, or vice versa. The difference in severity of edema due to age of animal, in this case, supports the observations of practical dairymen.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of the following: W. W. Yapp and E. E. Ormiston for judging the severity of udder edema; R. W. Touchberry for advice on the statistical treatment of the results, and R. L. Shirley and his staff for their care of the experimental animals.

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RELATIVE MERITS OF FIVE MEASURES OF A DAIRY SIRE'S TRANSMITTING ABILITY [1, 2]

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SUMMARY

Lactation milk and fat records of 6,949 daughters, 6,201 different dams, and the contemporary D.H.I.A. herd averages of 2,420 herds were studied, to compare five measures of a dairy sire's transmitting ability. They were the daughter average, daughter-dam difference, equal-parent index, daughter-contemporary herd difference, and daughter-contemporary-herd index. The daughters and dams were included in the D.H.I.A. proofs for 350 Guernsey and 486 Holstein sires used in artificial-breeding associations, and whose proofs were published for 1949–1951. A variance component analysis did not reveal any marked superiority for any of the measures, as determined by the percentage of the total variance comprised by the sire component for each of the measures. Sufficient data for the Holsteins were available for an empirical check of the reliability of these five measures. The simple daughter average appeared to be about as reliable as the equal-parent index or the daughter-herd index to predict future daughters' production for milk or fat. The D.H.I.A. herd average included the daughter in question and reduced the magnitude of the sire component for the herd measures. Use of a contemporary-herd average, which excludes the daughter in question in the two daughter-herd measures, should improve their merit as measures of a dairy sire's breeding value compared to the other measures in this study.

The performance of later daughters of sires selected for use in artificial-breeding associations, on the basis of proofs in one or two herds (natural), usually has reflected little of their sire's original apparent superiority *(10, 11)*. Much of the disappointment in the performance of the artificially sired daughters undoubtedly has resulted because conventional methods of evaluating sire proofs have not adequately accounted for the influence of herd differences and changes in intra-herd environment. The use of the contemporary herd average has been suggested to avoid this glaring weakness *(2, 8, 21)*. Most of the differences between herds are environmental *(15, 18, 19)* and, presumably, most of the intra-herd environmental changes are reflected by the herd average. Inasmuch as the herd average is readily available, could it be substituted for the dam's average in the daughter-dam difference and in the equal-parent index? If so, many more daughter-herd comparisons than daughter-dam comparisons would be permitted, since about one-half of the tested daughters have tested dams.

For these reasons, five measures of a sire's transmitting ability for milk and fat production, namely, the daughter average ($\bar{O}$), daughter-dam difference

Received for publication December 27, 1957.

[1] Contribution from the North Carolina Agricultural Experiment Station and published with the approval of the Director of Research as Paper No. 872 of the Journal Series.

[2] The data in this paper were taken from a dissertation presented by the senior author to the Graduate School, North Carolina State College, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1955.

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($\overline{O}$-$\overline{D}$), equal-parent index (2 $\overline{O}$-$\overline{D}$), daughter-contemporary herd difference ($\overline{O}$-$\overline{H}$), and daughter-contemporary herd index (2 $\overline{O}$-$\overline{H}$) were studied. The latter two measures can be visualized simply by substituting the actual herd average for the dam's average production in the daughter-dam difference and the equal-parent index.

EXPERIMENTAL PROCEDURE

Data. The data included lactation milk and fat records of 6,949 daughters, 6,201 different dams, and contemporary D.H.I.A. herd averages for 2,420 herds. The daughters and dams were included in the D.H.I.A. proofs of 350 Guernsey and 486 Holstein sires used in artificial-breeding associations in the United States, whose proofs were published for the years 1949–1951.

To be included in this study, each daughter had to have a contemporary D.H.I.A. herd average for at least one lactation and a dam with a record. The contemporary herd average used was the herd average completed the year following the year the individual cow freshened. This average was chosen after considering that many herds are on a reporting year which does not coincide with the calendar year, that the cow makes a record 10 mo. after freshening, and that the D.H.I.A. average is listed according to the year in which it is completed. Except for the D.H.I.A. herd average, all records were adjusted to a 305-day, 2 × milking, mature equivalent (M.E.) basis.

Naturally and artificially sired daughters of the sires were analyzed separately. The average production of the four groups studied is shown (Table 1).

TABLE 1
Production averages of groups in study

Group[a]	Daughters	Sires[b]	Herds	305 day, 2 ×, M. E. Milk	%	Fat
G–N	1,865	636	558	8,677	4.93	426
G–A	438	324	276	8,066	4.89	392
H–N	3,582	1,239	1,078	12,901	3.68	472
H–A	1,064	795	672	12,210	3.59	436

[a] G–N, Guernsey natural; G–A, Guernsey artificial; H–N, Holstein, natural; H–A, Holstein, artificial.
[b] These represent the number of sires on a within-herd and state basis. A total of 836 different sires was included, 350 Guernseys and 486 Holsteins.

The lower production level for the artificially sired daughters, as compared to the naturally sired animals, is typical of the situation throughout the country as reported from D.H.I.A. summaries *(11)*.

Analysis. Each of the five measures was expressed as a linear equation or model which included the effects of herd, sire-within-herd, and within-sire and herd effects on an intra-state basis. The sire-by-herd effect was not included, since previous analysis of similar data had shown it to be small *(13)* and the sire-by herd interaction variance could not be estimated with reasonable precision from these data. Using these models, the variances for each of the measures were expressed in terms of the components of variance for the simple daughter average. The details of procedure are described elsewhere *(6)*, but the resulting

expressions for the variances, pertinent to the interpretation of later results, are as follows:

(1) $$\sigma^2_{(\bar{o})} = \sigma^2_s + \frac{\sigma^2_h}{\text{No. of herds}} + \frac{\sigma^2_e}{\text{No. of daughters}}$$

(2) $$\sigma^2_{(\bar{o}-\bar{D})} = \sigma^2_s + \frac{2\,\sigma^2_h\,(1-r)}{\text{No. of herds}} + \frac{\sigma^2_e + \sigma^2\theta}{\text{No. of daughters}}$$

(3) $$\sigma^2_{(2\,\bar{o}-\bar{D})} = 4\,\sigma^2_s + \frac{\sigma^2_h\,(5-4r)}{\text{No. of herds}} + \frac{2\,\sigma^2_g + 5\,\sigma^2\theta}{\text{No. of pairs}}$$

(4) $$\sigma^2_{(\bar{o}-\bar{H})} = \sigma^2_s\left(1 - \frac{2n_{il.}}{n_{i..}} + \frac{n^2_{il.}}{n^2_{i..}}\right) +$$

$$\frac{\sum_{ij \neq 1} \frac{n_{ij.}{}^2\,\sigma^2_s}{n^2_{i.}} + 2\,\sigma^2_h\,(1-r)}{\text{No. of herds}} + \frac{\sigma^2_e\left(1 - \frac{1}{n_{i..}}\right)}{\text{No. of daughters}}$$

(5) $$\sigma^2_{(2\,\bar{o}-\bar{H})} = \sigma^2_s\left(4 - \frac{4n_{il.}}{n_{i..}} + \frac{n^2_{il.}}{n^2_{i..}}\right)$$

$$\frac{\sum_{ij \neq 1} \frac{n^2_{ij.}\,\sigma^2_s}{n^2_{i..}} + \sigma^2_h\,(5-4r) + \frac{\sigma^2_e\left(4 - \frac{3}{n_{i..}}\right)}{\text{No. of daughters}}}{\text{No. of herds}}$$

Where: σ_s^2 denotes the variance due to the additive genetic effects of the sire, possibly some epistatic effects (σ_i^2) and genetic environmental interactions.

σ_h^2 denotes the variance due to the genetic and environmental effects common to a herd but different from these effects on other herds.

σ_e^2 denotes the variance peculiar to the cow. This includes existing errors in measurement of the record and environmental influence not accounted for by the herd effect (σ^2) and the genetic effects not accounted for by the sire. Hence, $\sigma_e^2 = \sigma_d^2 + \sigma_i^2 + \frac{3\,\sigma_g^2}{4} + \sigma^2$ where σ_d^2 represents the dominance variance.

σ_g^2 denotes the variance due to differences in the additive breeding value of the individual cow and, as used, possibly includes some epistatic variance and genotype environmental interaction.

σ_θ^2 differs from σ_e^2 in that it does not include the additive genetic variance attributed under random mating to the dam and to chance at segregation ($\sigma_\theta^2 = \sigma_d^2 + \sigma_i^2 + \sigma^2$).

r is the correlation between the herd effects for the daughter and her dam or herd mates.

$n_{ii.}$ is the number of daughters of the sire under consideration in the ith herd.

$\sum_{ij \neq 1} n_{ij.}$ is the number of daughters of other sires in the ith herd.

Individual analyses of variance were computed for each of the five measures for milk and fat production for the two breeds, divided into natural and artificial groups. From each, estimates of the variance components were derived for herd, sire-within-herd, and within-herd and sire effects on an intra-state basis using Method I of Henderson *(7)*.

Since averages of records, differences, and indexes were used in the several measures, it was necessary to adjust the error variances to a single record basis, so that the results could be presented independent of the numbers of records per animal characteristic of these data. The formula used to adjust the error variance of an average of n records was

$$\sigma_e^2 = \sigma_{\bar{e}}^2 \frac{\overline{n}}{1 + (\overline{n} - 1)\, r_{dd}}$$

where $\overline{n}$ is the harmonic mean of the number of records per cow. The repeatability (r_{dd}) of successive records of a cow was taken as 0.50 for milk and 0.40 for fat production, as based on values from the literature *(1, 9, 12, 14, 20)*. The harmonic means in these data were as follows:

	Daughters	Dams
Guernsey, natural	1.32	2.18
Guernsey, artificial	1.22	2.08
Holstein, natural	1.32	2.27
Holstein, artificial	1.22	2.14

Similarly, the error variances for the other measures were adjusted to a single record or comparison basis with appropriate formulae being derived for each.

The fraction of the total estimated variance in each measure, which was represented by the sire component estimate, was computed. Since some of the sires had both naturally and artificially sired daughters, an empirical check was obtained of the values from the variance component analysis. Correlations for each of the five measures of a sire's transmitting ability were computed between naturally and artificially sired daughters and between a specific number of first and later artificially sired daughters for the Holstein data.

RESULTS AND DISCUSSION

Variance component analysis. Since the individual lactation milk and fat records are basic to the interpretation of the other measures, the importance of the several sources of variation in them is of primary concern. The components of variance for milk and fat for the daughter average are given in the second,

third, and fourth columns of Table 2. The percentages of the intra-state variance which they represent are given in the second column of Table 3.

Results comparable to those obtained in this study were secured by Legates *et al. (13)*, in an analysis of records of 5,234 Guernsey and 17,581 Holstein daughters of sires used in artificial-breeding associations. The Guernsey sire components accounted for 5.8 and 4.1% of the intra-state variance in milk and fat, respectively, in their data. The comparable percentages for the Holstein sire components were 7.7 and 7.8, respectively. In a study of New York Holstein data *(5)*, the sire component accounted for only 2.8% of the variance in lactation fat records. Carter *(3)* has since reported that adjustments for year, herd, and season effects removed much nongenetic variation. Following these adjustments, the sire component accounted for 7.7% of the variation.

The differences in the results for the Guernseys and the Holsteins in this study possibly could be explained by sampling, since the number of Guernsey sires was considerably less than the number of Holstein sires. However, lower values for the Guernsey sire components were also obtained in the study by Legates *et al. (13)*.

Values for the herd component of variance are in general agreement with those in the literature *(13, 16, 17)*. Herd differences generally accounted for a larger percentage of the total variance for the artificial groups.

Estimates of the variance components for all five measures for milk and fat are given (Table 2). The variances which are estimated are the functions of the components presented in the investigation for each measure, *e.g.*, for the daughter-dam difference, the σ_h^2 as computed estimates the expected $2\,\sigma_h^2$ (1-r). Inspection of the values (Table 2) reveals several circumstances which could be predicted on the basis of the formulae derived for the variances of the measures. The sire variance for the equal-parent index is about four times as large as the corresponding components for the daughter average; likewise, the sire variance for the daughter-herd index is about four times the corresponding component for the daughter-herd difference. The sire variances for the two measures using the herd average were consistently smaller than they were for their counterparts, the daughter-dam difference and the equal-parent index. This was to be expected, since the cow's own record was a part of the herd averages used.

The sire component for the daughter-dam difference was expected to be comparable in size to the component for the daughter average. Values for the daughter-dam difference are higher for the Holsteins. Time trends during the interval the daughters and dams made their records could have contributed to this; but, if they were present, they should have influenced the equal-parent index to as great an extent. The small number of degrees of freedom for sires, however, does not rule out the possibility that sampling may be responsible.

One criterion for evaluating a measure of a sire's transmitting ability is to require the measure to maximize sire differences, thereby permitting more accurate choices among sires. The extent to which this is accomplished can be expressed in terms of the fraction of the total variance which is represented by

TABLE 2

Estimates of variance components for all five measures

Group	Daughter			Daughter-dam			Equal-parent index			Daughter-herd			Daughter-herd index		
	Herd	Sire	Error[a]	Herd	Sire	Error[a]	Herd	Sire	Error[a]	Herd	Sire	Error[a]	Herd	Sire	Error[a]
							Milk								
G–N	9,734	890	20,535	5,722	2,418	37,096	24,662	4,502	94,732	4,414	525	20,628	19,338	2,633	81,641
G–A	3,067	9,701	15,136	3,317	7,685	33,099	−2,302	37,718	81,154	−20	10,969	15,357	−2,055	40,157	60,481
H–N	18,871	8,321	42,561	5,870	13,673	78,314	39,429	32,726	199,197	1,078	8,740	44,060	21,615	30,940	171,823
H–A	21,304	7,045	31,509	11,699	13,568	53,608	50,809	38,647	138,725	8,200	6,022	32,761	40,098	25,459	127,490
							Fat								
G–N	2,588	344	4,830	1,400	561	8,996	6,441	1,285	22,822	841	262	4,861	4,592	1,161	19,221
G–A	2,371	594	3,647	1,950	28	7,823	4,257	3,180	19,293	1,691	992	3,576	5,809	2,905	13,322
H–N	2,717	990	5,946	961	1,493	11,066	6,060	3,552	28,078	1,392	771	6,071	3,590	3,045	23,834
H–A	3,017	806	4,191	1,451	1,173	7,824	6,837	3,652	19,839	996	710	4,254	5,527	2,908	16,748

[a] Within-sire, within-herd—among paternal sisters in the same herd.

the sire component. Table 4 presents such a comparison for all five measures. Close inspection of this table reveals that no measure has emerged as superior. Although the percentages for the Guernsey natural group are markedly lower than those for the other three groups, there is no consistent trend in any one measure either for milk or for fat.

Using the variance components, repeatability values for each measure can be computed for any number of daughters or herds. For example, the repeatability of the daughter average

$$\sigma_s^2/\sigma_s^2 + \frac{\sigma_h^2}{\text{No. of herds}} + \frac{\sigma_e^2}{\text{No. of daughters}}$$

for fat for a Holstein sire with five artificially sired daughters in five herds is 0.36 or, for ten daughters in ten herds, 0.53. Usually, natural proofs include daughters in just one or two herds. For a Holstein sire with five daughters all in one herd, the repeatability of the daughter average for fat is 0.20 or for a sire with ten daughters all in one herd it is 0.23. In each case these values indicate the fraction of the superiority of a bull's first daughters that would be expected to show in a later group of daughters located in different herds. For the other measures considered, such values represent the fraction of the superiority based on that particular measure that one would expect to be exhibited by a later group of progeny, evaluated with that measure.

Much hope has been expressed for the two herd measures. Perhaps their sire components might have represented higher percentages of the total variance if the most contemporary herd average could have been secured in all cases.

Since the cow's own record could not be separated from the herd average, herd size also introduced another important factor. For herds smaller than the average of 24.2 cows for Holsteins and 26.4 cows for Guernseys in this study, the sire variance of these measures is reduced at an increasing rate as the herd size diminishes; whereas, the sire variance is reduced little for herds larger than those in the study. This is shown by the repeatability estimates for ten daughters in ten herds for the daughter-herd difference for fat production of the Holstein artificial group for 10, 20, 30, and 40 cow herds of 0.44, 0.54, 0.56 and 0.57, re-

TABLE 3

Percentage of the total variance which is represented by the sire component for each of the measures

Breed group	Daughter	Daughter-dam	2 Daughter-dam	Daughter-herd	2 Daughter-herd
			Milk		
G–N	2.9	5.3	3.6	2.1	2.5
G–A	34.8	17.4	31.7	41.7	39.9
H–N	11.9	14.0	12.1	16.2	13.8
H–A	11.8	17.2	16.9	12.8	13.2
			Fat		
G–N	4.4	5.1	4.2	4.4	4.6
G–A	9.0	.3	11.9	15.8	12.6
H–N	10.2	11.0	9.4	9.4	10.0
H–A	10.1	11.2	12.0	11.9	11.5

spectively. With the use of a contemporary herd average which excludes the daughter in question, the two daughter-contemporary-herd measures would be likely to give higher percentage values for the sire component than those in this study.

Empirical analysis. Correlations between each of the five measures of a sire's transmitting ability based on naturally or artificially sired daughters, and the performance of a specific number of future artificially sired daughters, were computed. For this analysis, all five of the naturally sired daughters were from the same herd, and each of the artificially sired daughters or comparisons for a sire were from a different herd. With these restrictions, only the Holstein groups had sufficient data to warrant analysis. Correlation values obtained from this phase of the study are given (Table 4).

While the correlations do not show a decisive margin in favor of any one of the measures, the simple daughter average appears to be almost as reliable as any of the other measures. The correlation values involving the daughter average, the equal-parent index, and the daughter-herd index are of approximately the same magnitude. The correlations for the daughter-herd difference were generally slightly lower than these three; whereas, those for the daughter-dam difference were lowest in seven of the eight comparisons. Use of a nonpaternal stablemate's average instead of the herd average probably would improve the merits of the two daughter-herd measures, because in the average- and small-sized herds a significant portion of the sire effect is removed when the herd average is subtracted from the daughter average.

The empirical correlations between the original natural proof and a later artificial proof were consistently lower than those for an original and a later artificial proof for the one comparable situation. This has been the experience of many in artificial breeding *(10)*. There was no real difference in the ranking of the measures between the artificial and the natural groups.

These results are in partial agreement with those of Carter *et al*, *(4)*. They found the daughter-dam difference and a daughter-contemporary difference to be

TABLE 4

Correlations between the measures of a sire's transmitting ability and the performance of his artificially sired daughters (Holsteins)

Variables[a]	No. of sires	Measure				
		Daughter	Daughter-dam	½ Daughter-dam	Daughter-herd	½ Daughter-herd
				Milk		
X_1 and X_2	39	0.20	−0.11	0.18	0.13	0.19
X_2 and X_3	47	0.28	0.08	0.30*	0.21	0.28
				Fat		
X_1 and X_2	39	0.26	0.15	0.24	0.27	0.32
X_2 and X_3	47	0.34*	0.16	0.35*	0.40**	0.38**

* Denotes statistical significance at 5% level of probability.
** Denotes statistical significance at 1% level of probability.
[a] X_1 = First five naturally sired daughters.
X_2 = First five artificially sired daughters.
X_3 = Next five artificially sired daughters.

about equal in merit. However, in their study the daughter average appeared to be of little value, and the equal-parent index was slightly less dependable than the daughter-dam and the daughter-contemporary comparisons.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to F. J. Verlinden and the staff of the Statistical Laboratory at North Carolina State College. They also wish to acknowledge the assistance of J. F. Kendrick of the Dairy Herd Improvement Section of USDA in assembling the data for the study.

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EFFECTS OF THYROTROPHIN, L-THYROXINE, AND L-TRIIODOTHYRONINE ON LEVELS OF PLASMA PROTEIN-BOUND IODINE IN DAIRY BULL CALVES[1, 2]

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SUMMARY

Intramuscular injections of thyrotrophin and intravenous injections of sodium L-thyroxine pentahydrate and sodium L-triiodothyronine were made in dairy bull calves (body wt. from 166 to 264 kg.), and plasma levels of protein-bound iodine (PBI) were used as a response criterion. The injection of thyrotrophin (Armour) in amounts of 10, 20, 40, and 80 U.S.P. units caused maximum PBI increases above initial levels ranging from 3.05 to 8.02 μg. % at 15 to 31 hr. postinjection. The mean biological half-life of the endogenously produced PBI, during its return to normal levels, was 22.1 hr. L-thyroxine (2.5 and 5.0 mg.) and L-triiodothyronine (5.58 and 11.16 mg.) were injected in each of three trials. Two first-order exponential regressions characterized the decline of PBI to normal in each instance. The first represented the rate of movement of the materials into their respective volumes of distribution (mean biological half-time of 1.93 hr. for L-thyroxine and 0.31 hr. for L-triiodothyronine). The second regression measured the rate of peripheral utilization or disposal of the materials (mean biological half-life of 18.0 hr. for L-thyroxine and 5.10 hr. for L-triiodothyronine). The mean volume of distribution for L-thyroxine was 9.07% of body wt.; whereas, the corresponding value for L-triiodothyronine was 37.5%. The constants developed in the L-thyroxine infusion trials were used to estimate the rate of thyroxine secretion or degradation (mean of 0.231 mg. per day per 100 lb. body wt.).

The thyroid gland accumulates iodide from the blood *(13, 16)*, synthesizes thyroglobulin *(2)*, and either stores thyroglobulin in the follicles of the gland or discharges thyroxine *(16)* into the circulation, all as the result of the activity of thyrotrophin from the anterior pituitary.

It has been determined that thyroxine is the major form of protein-bound organic iodine in the normal plasma *(2, 12, 15)* and in plasma following administration of thyrotrophin *(6)*. Both endogenous and exogenous thyroxine in the plasma are protein-bound in the same way, and have identical protein linkage *(5)*. Exogenous triiodothyronine also becomes protein-bound in the plasma *(3)*.

Rate constants have been presented for the disappearance of thyrotrophin-stimulated protein-bound iodine (PBI) *(10)* and, also, for the disappearance of plasma PBI resulting from exogenous thyroxine (*9, 14*). In addition, Ingbar and Freinkel *(7)* have presented a method for estimating the daily rate of thyroxine degradation which is equivalent to thyroxine secretion rate. This may be calculated utilizing as components the normal level of plasma protein-

Received for publication January 30, 1958.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Science, New Brunswick.

[2] This study was part of a Northeast Regional Project (NE-30, The Relation of Certain Physiological Factors to the Productive Capacity of Dairy Cattle), a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds of the United States Department of Agriculture.

bound iodine, the fractional rate of turnover per day of extrathyroidal thyroxine, and the thyroxine distribution volume or space.

It is the purpose of this paper (using dairy bull calves) to present the quantitative aspects of the stimulation of the thyroid as reflected in plasma level of PBI; to characterize the rate of disappearance or turnover rates of PBI from plasma after the intravenous injections of L-triiodothyronine and L-thyroxine, and to estimate the daily thyroxine secretion or degradation rates, using the principles proposed by Ingbar and Freinkel *(7)*.

MATERIALS AND METHODS

Two dairy bull calves (Guernsey 200[6] and Holstein 639[6]) were used in a series of separate experiments in which thyrotrophin (TH) was injected intramuscularly and L-thyroxine and L-triiodothyronine were injected intravenously. The thyrotrophin weighed 1.515 mg. per U.S.P. unit.[3] The L-thyroxine was in the form of sodium L-thyroxine pentahydrate[4] (mol. wt.—888.995, purity—96.2%); whereas, the L-triiodothyronine was in the form of sodium L-triiodothyronine[5] (mol. wt.—673.003, purity—92.4%).

Blood samples were taken initially and at varying intervals of time following the injection of the above materials and were assayed for plasma PBI by the method of Brown *et al. (1)*, as modified by Lennon and Mixner *(8)*. The PBI responses were calculated by subtracting the initial level of PBI from the subsequent levels of PBI in any given experiment.

RESULTS AND DISCUSSION

Thyrotrophin. In successive experiments, G200[6] was injected intramuscularly with 10 and 40 U.S.P. units of thyrotrophin and H639[6] was injected with 20 and 80 U.S.P. units of thyrotrophin. The nature of the PBI responses, as a function of the time after injection of the thyrotrophin, is presented as a semilogarithmic plot (Figure 1). In three of the four trials, maximum levels of PBI were reached at about 15 hr. after injection, and thereafter declined at a rather steady rate.

The quantitative aspects of the thyrotrophin-PBI response curves (Figure 1) may be analyzed by the methods outlined by O'Neal and Heinbecker *(10)* for thyrotrophin stimulation of PBI in hypophysectomized dogs. These analyses are summarized (Table 1). It is suggested that the declining phase of PBI from the plasma is owing entirely to the disappearance of PBI from the plasma and is best described by a simple exponential regression having the general formula, $PBI = ae^{-bt}$. This regression may be calculated by the least squares method, in which X-values are time in hours after injection of thyrotrophin and Y-values are the natural logarithms of the PBI values in μg. %.

[3] Thyrotrophin, Lot R491031, was generously supplied by Dr. Irby Bunding, The Armour Laboratories, Chicago.

[4, 5] Supplied through the courtesy of Dr. H. L. Saunders, Smith, Kline and French Laboratories, Philadelphia.

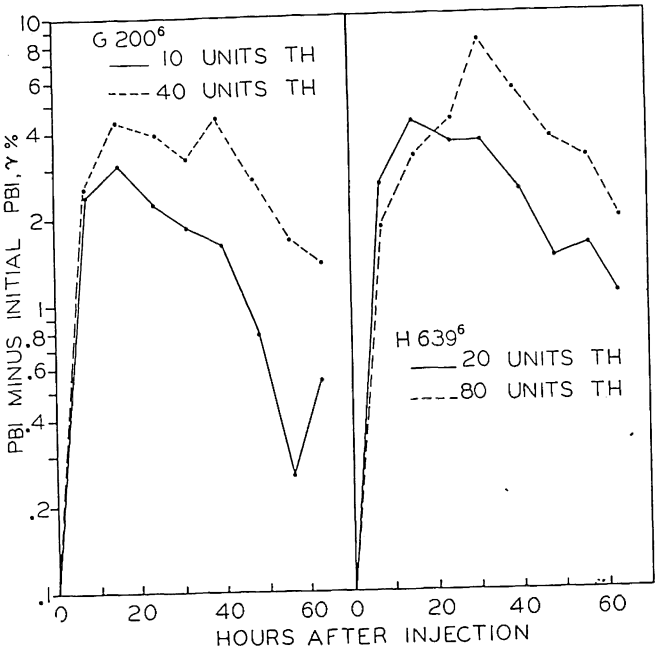


FIG. 1. Semilogarithmic plot of the PBI responses of dairy bull calves injected intramuscularly with varying doses of thyrotrophin (Armour).

TABLE 1

Summary of certain PBI response characteristics to thyrotrophin injected intramuscularly in two dairy bull calves

Characteristic	Animal				Means
	G200[6]		H639[6]		
Age *(mo.)*	5.4	6.1	4.9	5.6	
Body weight *(kg.)*	166	188	189	218	
Normal PBI *(μg. %)*	3.06	4.23	3.36	4.08	3.68
Dosage TH *(U.S.P. units)*	10	40	20	80	
Maximum stimulation of PBI above normal level *(μg. %)*	3.05	4.38	4.22	8.02	
Time to reach maximum PBI *(hr.)*	15±4	15±4	15±4	31±4	19
PBI disposal curve					
1. Degrees of freedom *(n−2)*	8	5	8	3	6
2. Coefficients of correlation between time in hours after thyrotrophin administration and the natural logarithms of PBI *(μg. %)*	−0.93[a]	−0.88[a]	−0.94[a]	−0.99[a]	−0.94[a]
3. Intercept of curve on Y-axis at zero-time *(PBI-μg. %)* (a)	7.86	7.48	6.11	28.39	
4. Calculated total amount of thyroxine stimulated by TH, based on thyroxine space or volume of distribution of 9.07% of body wt. *(mg.)*	1.812	1.953	1.604	8.596	
5. Slope of rate constant of regression of PBI to normal levels $(-b_2)$ *(per hour)*	0.0485	0.0244	0.0240	0.0416	0.0346
6. Half-life of PBI [*hr.* $(t_{1/2})$]	14.3	28.4	28.9	16.7	22.1

[a] Significant at the 1% level ($P < 0.01$).

By use of the regression and its associated constants, five characteristics concerned with PBI disposal from the plasma may be calculated (Characteristics 2 to 6, Table 1).

Characteristic 2 is the coefficient of correlation *(r)* between the time in hours after thyrotrophin injection (*X*-variable) and the natural logarithms of the PBI levels in μg. % (*Y*-variable) during the declining or disposal phase of PBI response. The mean coefficient of correlation for the four trials was −0.94 (Table 1), which is considered to be satisfactorily high.

The *a* constant of the general equation (Characteristic 3, Table 1) is the *Y*-axis intercept of the regression line projected back to zero-time. This is an estimate of the total amount of PBI which has been stimulated by the thyrotrophin per 100 ml. of plasma, or per 100 ml. of the distribution volume of thyroxine.

By the use of constant *a* (Characteristic 3), an estimate of the total amount of thyroxine which may be stimulated by a single injection of thyrotrophin (Characteristic 4) may be calculated. In the next section, it was estimated that the mean L-thyroxine space or volume of distribution was equivalent to 9.07% of body wt. Since PBI is nearly all thyroxine iodine, the total amount of thyroxine stimulated by the thyrotrophin may be estimated by the formula:

$$\text{total thyroxine stimulated, } \mu\text{g.} = \frac{\text{a}/0.653 \times \text{V.D.}}{100}$$

where *a* = intercept of PBI response curve on *Y*-axis at zero-time, PBI, μg. %
0.653 = fraction of thyroxine which is iodine by weight
V.D. = volume of distribution of L-thyroxine in ml. = body wt., g. × 0.0907

From Table 1 (Characteristic 4), the estimated total amounts of thyroxine stimulated by thyrotrophin ranged from 1.604 to 8.596 mg. Although the correspondence between total amounts of thyroxine stimulated and the doses of thyrotrophin injected was not good in the four trials, it did provide some useful estimates.

The mean slope value of rate constant *b* (Characteristic 5, Table 1) for the decline in PBI with time in the four trials was 0.0346; that is, a mean decline of 3.46% per hour in the PBI levels on an instantaneous basis. The slope or rate constants *(b)* may be more easily interpreted when presented as biological half-life values $t_{1/2}$ (Characteristic 6, Table 1), which are calculated by dividing 0.693 (natural logarithm of 2) by *b*, the slope or rate constant value. The biological half-life for PBI stimulated by thyrotrophin ranged from 14.3 to 28.9 hr., with a mean of 22.1 hr., which is similar to the mean biological half-life of 18.0 hr. obtained for PBI from exogenous L-thyroxine (next section and Table 2, disposal curve). O'Neal and Heinbecker *(10)* obtained a mean biological half-life of 16.2 hr. for the decline in PBI after the injection of a single dose of thyrotrophin in hypophysectomized dogs.

TABLE 2

Distribution and disposal characteristics of L-*thyroxine injected intravenously in dairy bull calves as measured by plasma PBI*

	H639[a]	G200[a]	G200[a]	Means
Age *(mo.)*	7.4	6.2	7.6	
Body weight *(kg.)*	264	233	198	
Normal PBI *(μg. %)*	3.47	4.29	4.07	3.94
Dosage hormone *(mg.)*				
Actual	2.86	2.86	5.72	
L-thyroxine equivalent	2.50	2.50	5.00	
Distribution curve (B)				
Degrees of freedom ($n-2$)	6	5	3	4.5
r^a	−0.91[b]	−0.95[b]	−0.99[b]	−0.95[b]
a_1 *(PBI-μg. %)*	4.71	4.25	18.88	
$-b_1$ *(per hour)*	0.302	0.262	0.806	0.457
$t_{1/2}$ *(hr.)*	2.29	2.65	0.86	1.93
Disposal curve D				
Degrees of freedom ($n-2$)	6	6	10	7.3
r^a	−0.98[b]	−0.98[b]	−0.98[b]	−0.98[b]
a_2 *(PBI-μg. %)*	6.57	8.63	17.07	
$-b_2'$ *(per hour)*	0.0406	0.0417	0.0343	0.0389
$-b_2^2$ *(per day)*	0.9744	1.0008	0.8232	0.9336
$t_{1/2}$ *(hr.)*	17.1	16.6	20.2	18.0
Volume of distribution				
Liters	24.9	18.9	19.1	
% Body weight	9.42	8.13	9.66	9.07
Thyroxine secretion per day				
Total *(mg.)*	1.285	1.242	0.995	
Per 100 lb. body wt. *(mg.)*	0.222	0.243	0.229	0.231

[a] Coefficient of correlation between time in hours after injection of thyroxine *(X)* and $\log_e$ of PBI concentration, μg. %, *(Y)* of the first-order regressions.
[b] Significant at the 1% level ($P < 0.01$).

TABLE 3

Distribution and disposal characteristics of L-*triiodothyronine injected intravenously in dairy bull calves as measured by plasma PBI*

Characteristic	H639[a]	H639[a]	G200[a]	Means
Age *(mo.)*	6.2	7.3	7.8	
Body weight *(kg.)*	232	255	216	
Normal PBI *(μg. %)*	3.84	4.39	4.79	4.34
Dosage hormone *(mg.)*				
Actual	5.777	11.554	11.554	
L-triiodothyronine equivalent	5.580	11.160	11.160	
Distribution curve (B)				
Degrees of freedom ($n-2$)	1	5	6	4.0
r^a	−0.999[b]	−0.98[b]	−0.99[b]	−0.99[b]
a_1 *(PBI-μg. %)*	8.48	16.82	18.50	
$-b_1$ *(per hour)*	1.92	2.47	2.42	2.27
$t_{1/2}$ *(hr.)*	0.36	0.28	0.29	0.31
Disposal curve (D)				
Degrees of freedom ($n-2$)	3	10	7	6.7
r^a	−0.99[b]	−0.93[b]	−0.96[b]	−0.96[b]
a_2 *(PBI, μg. %)*	3.97	7.13	7.35	
$-b_2$ *(per hour)*	0.135	0.134	0.139	0.136
$t_{1/2}$ *(hr.)*	5.13	5.17	4.99	5.10
Volume of distribution				
Liters	82.3	91.7	88.9	
% Body weight	35.5	36.0	41.1	37.5

[a] Coefficient of correlation between time in hours after injection of triiodothyronine *(X)* and $\log_e$ of PBI concentration, μg. %, *(Y)* of the first-order regressions.
[b] Significant at the 1% level ($P < 0.01$).

L-thyroxine and L-triiodothyronine. In a series of trials, the two bull calves were injected intravenously with sodium L-thyroxine pentahydrate (Table 2) and sodium L-triiodothyronine (Table 3) in varying quantities. Blood samples were collected at intervals over a period of approximately 72 hr. after injection in the L-thyroxine series and 12 hr. in the L-triiodothyronine series, and were assayed for PBI.

In both series of experiments, the levels of PBI declined rapidly during the initial phase following the injection of the materials and less rapidly thereafter. The methods of O'Neal *(9)* for the analysis of composite curves were applied to the various groups of experimental PBI-time series data.

The curves were each shown to have two components, each of which may be expressed as a first-order regression with an exponential rate of decline in which $\log_e$ of PBI concentration in the plasma was rectilinearly and inversely related to the time after the injection of the materials (Figure 2). The initial rapid decline in PBI, as represented by the initial first-order regressions (B curves, Figure 2), may be due to the distribution of thyroxine from the plasma into a larger body fluid compartment or space (volume of distribution). The slower decline in PBI concentrations, as represented by the second first-order regressions (D curves, Figure 2), may be due to the peripheral metabolism or disposal of the materials after distribution has been completed. Figure 2 presents representative trials for L-thyroxine and L-triiodothyronine on G200[6], in which the two first-order regressions are presented in each instance. The biological half-

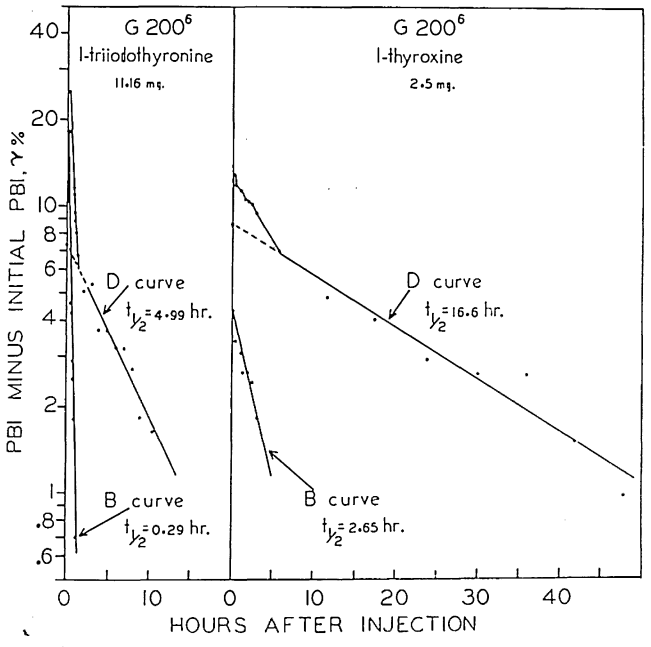


FIG. 2. Analysis of the curves of disappearance of PBI from the blood after the rapid injection of L-triiodothyronine (11.16 mg.) and L-thyroxine (2.5 mg.) in a Guernsey bull calf.

times ($t_{1/2}$) for distribution curves B were 0.29 and 2.65 hr. for L-triiodothyronine and L-thyroxine, respectively; whereas, the corresponding half-lives ($t_{1/2}$) for the disposal curves D were 4.99 and 16.6 hr., respectively.

The mathematical model for the disappearance of PBI from plasma is as follows:

$$\text{PBI, } \mu\text{g. \%} = a_1e^{-b_1t} + a_2e^{-b_2t}$$

in which a_1 = concentration of PBI (μg. %) at zero-time by extrapolation of the more rapid initial distribution curve,

$-b_1$ = slope (rate constant) of the distribution B curve, per hour,

a_2 = concentration of PBI (μg. %) at zero-time by extrapolation of the slower disposal D curve,

$-b_2$ = slope (rate constant) of the disposal D curve, per hour,

e = base of natural logarithms,

t = time in hours from injection of materials.

The biological half-life ($t_{1/2}$) for either of the rate reactions is calculated by dividing 0.693, the natural logarithm of 2, either by b_1 or by b_2, the rate constants.

Since a_2 equals the theoretical concentration of PBI in its volume of distribution at zero-time before any disposal has taken place, the volume of distribution (V.D.) of either thyroxine or triiodothyronine may be calculated by the following formula:

$$\text{V.D., ml.} = \frac{\text{dose } (\mu\text{g.}) \text{ of material} \times 100}{a_2/k_1 \text{ or } k_2}$$

in which k_1 = fraction of thyroxine (0.693) which is iodine by wt.

and k_2 = fraction of triiodothyronine (0.586) which is iodine by wt.

A summary of the kinetic characteristics for the distribution and disposal of L-thyroxine (Table 2) and L-triiodothyronine (Table 3) is presented for the two animals in the series of trials. In all instances, the coefficients of correlation *(r)* between the time in hours after injection of materials and the $\log_e$ of PBI of the several first-order regressions were high (range, −0.91 to −0.99), indicating that the mathematical model and the chemical analysis for PBI were satisfactory.

The rapidity with which L-thyroxine moved into its volume of distribution (mean distribution half-time, $t_{1/2}$, of 1.93 hr., Table 2) was much slower than the corresponding time for L-triiodothyronine (mean distribution half-time, $t_{1/2}$, of 0.31 hr., Table 3). O'Neal *(9)* reported a distribution half-time for D, L-thyroxine in dogs of 1.29 hr.

The mean disposal half-life for L-thyroxine ($t_{1/2}$, 18.0 hr., Table 2) was much longer than the mean disposal half-life for L-triiodothyronine ($t_{1/2}$, 5.1 hr., Table 3). The 22.1 hr. half-time of PBI stimulated by thyrotrophin (Table 1, Characteristic 6) was very similar to the 18.0 hr. disposal half-life for exogenous L-thyroxine (Table 2), which was an indication that the PBI in these instances

was primarily thyroxine iodine as contrasted to L-triiodothyronine iodine, which had a PBI disposal half-life of only 5.1 hr. O'Neal *(9)* found a disposal half-life of 24.6 hr. for D,L-thyroxine injected in dogs, and Recant and Riggs *(11)* reported a disposal half-life of PBI after injection of D,L-thyroxine in a normal human subject to be 4.3 days, or 103.2 hr.

The mean volumes of distribution for L-thyroxine (Table 2) and L-triiodothyronine (Table 3) were 9.07 and 37.5% of body wt., respectively. Triiodothyronine compared to thyroxine had a larger volume of distribution, faster rates of distribution and disposal, and lower iodine content, all reasons why L-triiodothyronine results in lower plasma levels of PBI in calves. O'Neal *(9)* reported that the volume of distribution of thyroxine in dogs was 36.9% of body wt., and Feldman *(4)* reported that the volumes of distribution for labeled thyroxine and triiodothyronine in the rat were 13 and 26%, respectively.

The method of Ingbar and Freinkel *(7)* was used to calculate the daily rate of thyroxine secretion (or degradation) of the two experimental animals (Table 2), using the data obtained in the three L-thyroxine infusion trials. These were calculated by multiplying the original PBI level by the volume of distribution of thyroxine by the $-b_2^2$ slope factor (fractional rate of thyroxine turnover), using the proper units in each instance, expressed on a daily basis. The daily rate of thyroxine turnover ($-b_2^2$) was obtained by multiplying the hourly rate of thyroxine turnover ($-b_2'$) by 24. The turnover values expressed as PBI were calculated in terms of thyroxine by dividing the completed calculations by 0.653, the fractional weight of iodine in thyroxine. The estimated amounts of thyroxine secreted (or degraded) by the animals per 100 lb. body wt. per day varied from 0.222 to 0.243 mg., with a mean of 0.231 mg. These trials were performed during the summer months (August and September), and the estimated daily thyroxine secretion rates agree well with those reported by Premachandra *et al. (11)* for cattle in summer. This is the first report presenting a practical method for determining thyroxine secretion rates in cattle which does not require the use of radioisotope facilities; therefore, it might have more extensive application.

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THE RATE OF MACHINE MILKING OF DAIRY COWS. II. EFFECT OF VACUUM AND PULSATION RATE[1]

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SUMMARY

Eighteen randomly selected slow-milking cows were milked with a machine operated at various vacuum levels (10, 12.5, and 15) and pulsation rates (20, 50, and 80) for a 27-day experimental period. The nine treatments were assigned at random at the start of each of the three replications included in the above experimental period. Increasing the vacuum level increased the speed of milking and the effect was greater from 10 to 12.5 than from 12.5 to 15 in. Increasing the pulsation rate increased the speed of milking, but the magnitude of its effect was much less than that of increasing the vacuum. Most of the effect of pulsation on increasing the speed of milking occurred from 20 to 50. Increasing the pulsation rate appeared to be more important at low levels of vacuum than it was at high-vacuum levels. Most of the effects of vacuum and pulsation on increasing milking rate were additive. A few treatment interaction terms were significant. In these cases the increases in milking rate expected, had the effects of vacuum and pulsation been additive, were not obtained as a result of this interaction. Changing the vacuum level or pulsation rate affected the cows differently. In general, increasing the vacuum or pulsation caused greater increases in milking rate, percentage-wise, for the slowest-milking group of cows than it did for the fastest-milking group of cows.

It was evident from the studies of Dodd *(4)*, Beck *et al. (2)*, and the authors *(8)* that a few cows substantially slow up the milking routine. If these cows could be milked faster, it would result in a great time-saving for most dairymen.

Smith and Petersen *(6)*, Baxter *et al. (1)*, and Gregoire *et al. (5)* obtained increased rates of milk flow upon increasing the vacuum level. Smith and Petersen noted considerable cow-to-cow variation in reaction to the increased vacuum. Baxter *et al. (1)* stated that the vacuum not only affected cows differently, but that increased vacuum caused greater increases in peak flow for fast-milking cows than for slow-milking cows. However, neither Baxter *et al. (1)* nor Whittleston and Verrall *(10)* obtained a decrease in over-all milking rate as they increased the vacuum, because longer stripping times were required.

Whittleston *(9)* observed that pulsation action was in some cases necessary for stimulation of milk flow, but when the pulsator was stopped after a normal start, the cows continued to milk at a normal rate in most cases. Whittleston and Verrall *(10)* found no difference in average rate of milking using 21 and 42 or 42 and 84 pulsations per minute and 14¾ in. of vacuum. On the other hand, Clough *et al. (3)* obtained faster milking with each increase in pulsation rate between 20 and 80.

None of these experiments was specifically directed at the slow-milking cow problem, nor was any designed to determine the interaction, if any, between

Received for publication July 18, 1957.

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vacuum level and pulsation rate. Furthermore, the effects reported on increasing vacuum level and pulsation rate were somewhat inconsistent. The purpose of this paper is to report the results of an investigation which was designed to clarify and add to our knowledge on this subject. Part of these data has been presented previously in abstract form *(7)*.

EXPERIMENTAL PROCEDURE

A standard single-unit, Sterling-type De Laval milker suspended from a spring-type scale was used throughout this study. The scale had been fitted into the frame of a small wooden stand which could be placed between the cows at milking time.

A small weight-controlled vacuum pump supplied the vacuum for the machine. The three vacuum levels used, 10, 12.5, and 15 in. of mercury, were obtained by changing the weights in the vacuum pump. The three pulsation rates used, 20, 50, and 80 per minute, were obtained by setting the adjustable Sterling-type pulsator before each milking.

All cows were milked at their regular milking time. Each udder was cleaned and massaged with an individual towel dipped in warm chlorine solution. This was immediately followed by the use of the strip cup. The machine was applied as soon as let-down had occurred. When the last cup was in place, the stop watch was started. Scale readings were taken at 15-sec. intervals until the milk flow fell to 0.3 lb. (0.2 for one cow) or less between readings. At this point, machine-stripping was started and continued until the cessation of flow; whereupon, the stop watch was stopped and the machine removed.

Eighteen slow-milking cows, which included two Brown Swiss, one Guernsey, and 15 Holsteins, were selected randomly for this experiment. Of these, six were milked during the winter, seven during the spring, and five during the summer months. Each of the cows required a longer total milking time than the average cow of its respective breed at the same stage of lactation. Each cow was milked for a three-day preliminary period before the 27-day experimental period started. This allowed the cows a chance to become accustomed to the special equipment and attention, and gave the operator a chance to become acquainted with the individual cow.

Before the start of the experiment, the cows were milked with a standard pail-type De Laval milker operated at 12.5 in. of vacuum and 48 pulsations per minute. During the preliminary period and the experimental period they were milked once a day with a machine operated as above and once a day with the experimental apparatus.

The rates of milking measurements used were as follows: *(1)* machine time (time to start of machine stripping), *(2)* stripping time, *(3)* stripping yield, *(4)* total milking time, *(5)* total yield, *(6)* average rate of flow, *(7)* maximum rate of flow (fastest minute), and *(8)* time to maximum rate of flow.

The design of the experiments is outlined (Table 1). It consisted of a 3×3 factorial arrangement of treatments. The resulting nine treatments were ran-

TABLE 1
Design of experiment[a]

Vacuum levels	Pulsation rates		
	20	50	80
10	11[b]	21	31
12.5	12	22	32
15	13	23	33

[a] All cows were milked with the same treatment on a given day.
[b] Treatment code numbers.

domly assigned at the beginning of each of the three replications. This provided three milkings for each of the 18 cows with each treatment, or a total of 54 milkings with each of the nine treatments.

For the analysis of variance the average of the three replications was used. The data were then analyzed according to the variance component model, X_{ijk}[3] = $\mu + V_i + P_j + C_k + VP_{ij} + VC_{ik} + PC_{jk} + VPC_{ijk}$, in which V and P were considered fixed and C was considered a random variable (V = vacuum, P = pulsation rate, C = cow). Individual degrees of freedom were assigned to V and P, to determine if the differences between treatment means were linear, quadratic, or both. Individual degrees of freedom were also assigned to the various components of between-treatment interaction, VP.

RESULTS

Statistical analyses were carried out on all the rate-of-milking measurements. The treatment, individual vacuum level, and pulsation rate, and experimental averages for all the rate-of-milking measurements are given (Table 2).

Effect of vacuum level. The right-hand column (Table 2) contains the average values obtained for each vacuum level. Machine time decreased from 6 min., 10 sec. at 10 in., to 5 min., 9 sec. at 12.5 in., to 4 min., 34 sec. at 15 in. The decrease from 6 min., 10 sec. to 4 min., 34 sec. was highly significant, linearly. Further study of the individual cow data showed that the slowest-milking group of cows was affected the most by the vacuum increase, which suggests that their stronger sphincter muscles played a role in determining milking rate. This observation is supported by the work of Baxter *et al. (1)*. They found that inserting cannulas of constant bore into the ends of the teats just prior to applying the machine caused the resultant maximum rate of milk ejection to be similar for all cows.

Stripping time decreased from 67 to 53 sec. as the vacuum increased from 10 to 12.5 in. No additional decrease was obtained at 15 in., however. This plateauing effect caused a significant quadratic trend. The amount of milk from machine-stripping was not significantly affected. Total milking-time decreased significantly. This trend was both linear and quadratic.

Total yield was found to increase linearly as the vacuum increased. This

[3] X_{ijk} is the observation on the kth animal at the jth pulsation rate and the ith vacuum level.

TABLE 2

Effect of different vacuum levels and pulsation rates on milking rate: Average rates for 18 slow-milking cows. Each cow was milked three times with each treatment

	Vacuum levels	Pulsation rates 20	50	80	$\bar{x}$
Machine time *(min. and sec.)*	10	6:49	5:58	5:42	6:10
	12.5	5:31	5:04	4:53	5:09
	15	4:54	4:26	4:23	4:34
	$\bar{x}$	5:45	5:09	4:59	5:18
Significant: V_l[a], P_l[a], P_q[a], $V_l \times P_l$[a]					
Stripping time *(sec.)*	10	75	59	67	67
	12.5	51	54	55	53
	15	58	50	55	54
	$\bar{x}$	62	54	59	58
Significant: V_l[b], V_q[b]					
Stripping yield *(lb.)*	10	1.98	1.76	2.05	1.93
	12.5	1.70	2.03	2.10	1.94
	15	2.41	2.17	2.44	2.34
	$\bar{x}$	2.03	1.99	2.20	2.07
Significant: None					
Total milking time *(min. and sec.)*	10	8:04	6:57	6:50	7:17
	12.5	6:22	5:57	5:48	6:03
	15	5:50	5:16	5:18	5:28
	$\bar{x}$	6:45	6:03	5:59	6:16
Significant: V_l[a], V_q[a], P_l[a], P_q[a], $V_l \times P_l$[a]					
Total yield *(lb.)*	10	20.4	19.5	20.2	20.0
	12.5	20.7	20.9	20.4	20.7
	15	21.7	21.4	20.9	21.3
	$\bar{x}$	20.9	20.6	20.5	20.7
Significant: V_l[a], P_l[b], $V_l \times P_q$[b]					
Average rates of flow *(lb/min)*	10	2.6	2.9	3.1	2.9
	12.5	3.3	2.6	3.6	3.5
	15	3.8	4.1	4.0	4.0
	$\bar{x}$	3.3	3.5	3.6	3.4
Significant: V_l[a], V_q[b], P_l[a], P_q[a], $V_l \times P_l$[b]					
Maximum rate of flow *(lb/min)*	10	3.9	4.1	4.4	4.1
	12.5	4.8	5.3	5.3	5.2
	15	5.5	6.0	6.0	5.9
	$\bar{x}$	4.7	5.1	5.2	5.0
Significant: V_l[a], V_q[b], P_l[a], P_q[b], $V_l \times P_q$[b]					
Time to maximum rate of flow *(sec.)*	10	152	107	112	124
	12.5	103	91	83	93
	15	84	66	67	72
	$\bar{x}$	113	88	88	96
Significant: V_l[a], V_q[a], P_l[a], P_q[b]					

V—Vacuum; P—Pulsation; l—linear; q—quadratic.
[a]Significant at 1% level.
[b]Significant at 5% level.

may have been owing to the slow milking, which tended to allow the effect of oxytocin to wear off before the cows were completely milked.

Maximum rate of flow increased from 4.2 at 10 in. to 5.2 at 12.5 in. to 5.8 at 15 in. This trend also was both linear and quadratic.

Effect of pulsation rate. Average values obtained for each pulsation rate are shown (Table 2). In general, increasing the pulsation rate significantly increased the speed of milking, but not as much as did increasing the vacuum

level. This effect of increasing pulsation rate was most marked on increasing it from 20 to 50. No noticeable effect occurred on increasing the rate from 50 to 80. This means that 20 pulsations per minute was definitely too slow for all the vacuum levels used. This fact is emphasized by Figure 1. In this figure, the average total milking time of each treatment is represented by a dot. It

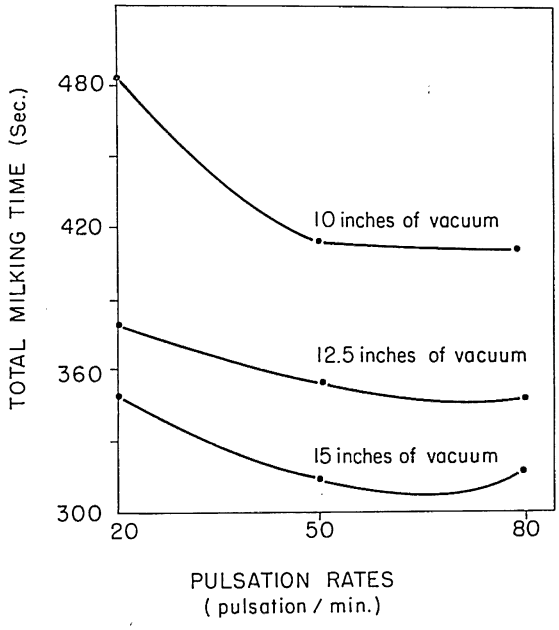


FIG. 1. Effect of different vacuum levels and pulsation rates on total milking time.

can be seen that increasing the pulsation rate from 20 to 50 was more important at the low level of vacuum than at higher ones.

Effect of interaction between different vacuum levels and pulsation rates. By assigning individual degrees of freedom to the various components of between-treatment interaction, it was possible to determine which components were important. These are enumerated (Table 2). Actually, few were statistically significant, indicating that the effects of vacuum and pulsation on milking rate are primarily additive.

The vacuum linear by pulsation linear component, $V_1 \times P_1$, was highly significant for machine-time and total milking time, and significant, $P < .05$, for average rate of flow. In these cases, the significance occurred because the combination of vacuum and pulsation resulted in slower milking than expected, based on the additive effects of vacuum and pulsation.

Effect of vacuum and pulsation on different cows. Great variation was found between the 18 slow-milking cows in their rate of milking. In fact, the magnitude of the between-cow mean squares indicated that this was the major source of variation in most of the analyses.

The various levels of vacuum affected the individual cows differently. This conclusion is based on the fact that the vacuum by cow interaction, VC, was highly significant for all the rate-of-milking measurements except stripping time, which was significant ($P < .05$). The three pulsation rates also affected the cows differently, since the pulsation-by-cow interaction, PC, was highly significant for total milking time, average rate of flow, and maximum rate of flow.

DISCUSSION

As was found by Smith and Petersen *(6)*, Baxter *et al. (1)*, and Gregoire *et al. (5)*, this study showed that as the vacuum increased the maximum rate of flow increased. As to magnitude of increase, Smith and Petersen *(6)* obtained a 100% increase in peak flow (maximum rate of flow) upon increasing the vacuum from 10 to 15 in. In this study, the maximum rate increased by only 40.5% as the vacuum increased from 10 to 15 in. Smith and Petersen found that more of the increase occurred between 10 and 12.5 than between 12.5 and 15, which is what occurred in this study.

Baxter *et al. (1)* reported that they failed to obtain an increase in average rate of flow as they increased the vacuum from 11 to 20 in., owing to the longer time necessary to remove the large amounts of strippings left at the higher vacuum levels. Whittleston and Verrall *(10)* also obtained no significant differences in average rate of flow between 10 and 14¾ or between 14¾ and 19 in. of vacuum. However, in our study, the average rate increased at approximately the same rate as the maximum rate of flow, 37.8, as compared to 40.5%.

Smith and Petersen *(6)* secured a lower amount of machine strippings at 14 in. of vacuum than at 10, 12, or 16 in. of vacuum, and Gregoire *et al. (5)* obtained smaller amounts of machine-strippings at 13 than at 10 or 17 in. of vacuum. In this study, there were no significant differences in amount of strippings between the three vacuum levels used, although the largest amount of strippings was obtained at 15 in. of vacuum. Gregoire *et al. (5)* also reported no significant differences in stripping time; whereas, in this experiment, the stripping-time decreased as the vacuum increased from 10 to 12.5. This is attributed to the fact that more pull could be applied to the teat cups while machine-stripping as the vacuum was increased. However, as the vacuum increased from 12.5 to 15, the stripping-time tended to remain about the same because of the somewhat larger amounts of strippings obtained at 15 in.

Gregoire *et al. (5)* found no significant difference in total yield over a 40-wk. period between 10, 13, and 17 in. of vacuum on 12 first-calf heifers. The 18 slow-milking cows in this study, however, showed a highly significant increase in total yield as the vacuum increased. Apparently, at the lower vacuum levels the effects of the oxytocin had begun to wear off because of the slow milking, before some of the cows were completed.

Similar to the findings of Smith and Petersen *(6)* and Baxter *et al. (1)*, great cow-to-cow variation was noted in the reaction of the cows to the various vacuum levels. Baxter *et al. (1)* found that the two fastest-milking cows had a

greater increase in maximum rate of flow, as the vacuum increased, than did the two slowest-milking cows in their study. A similar phenomena was noted in this study; although, percentage-wise, the slow-milking cows increased much more than did the faster-milking cows. The average and maximum rates of flow are compared for the four fastest- and four slowest-milking cows (see Table 3).

TABLE 3
Comparison of the average and maximum rates of flow for the four slowest and the four fastest cows at different vacuum levels and pulsation rates

Vacuum level	Average rate *(lb/min)*		Maximum rate *(lb/min)*	
	4 Slowest cows	4 Fastest cows	4 Slowest cows	4 Fastest cows
10	1.53	4.26	2.04	6.60
12.5	2.12	4.85	2.77	7.52
15	2.50	5.28	3.26	8.62
Diff. 10–12.5 inc.	0.59	0.59	0.73	0.92
	38.5%	13.9%	36.0%	14.0%
Diff. 10–15 inc.	0.96	1.02	1.23	2.02
	62.7%	24.0%	60.3%	30.6%

Pulsation rate	Average rate *(lb/min)*		Maximum rate *(lb/min)*	
	4 Slowest cows	4 Fastest cows	4 Slowest cows	4 Fastest cows
20	1.91	4.55	2.48	7.45
50	2.12	4.90	2.81	7.62
80	2.11	4.94	2.78	7.68
Diff. 20–50 inc.	0.21	0.36	0.33	0.17
	11%	8%	13%	2%
Diff. 20–80 inc.	0.19	0.39	0.30	0.23
	10%	9%	12%	3%

The magnitude of the increase in average rate is about the same for both groups but, percentage-wise, the increase is much greater for the slower-milking cows.

Whittleston and Verrall *(10)* noted that the teat cups tended to fall off more when 10 in. was used than when higher vacuums were used. A similar observation was noted in this study.

Whittleston and Verrall *(10)* found no statistically significant increase in milking rate upon increasing the pulsation rate from 21 to 42 or from 42 to 84 with the vacuum at 14¾ in. However, Clough *et al. (3)* obtained an increase in milking rate on increasing the pulsation from 20 to 80 at 15 in. of vacuum. Increasing the pulsation from 20 to 80 also resulted in faster milking in this study. Clough obtained a 1.39 lb.-per-minute increase in peak flow and a 0.34 lb.-per-minute increase in average rate of flow upon increasing the pulsation from 20 to 80. In this experiment, the maximum rate increased by 0.50 lb. per minute and the average rate of flow by 0.31 lb. per minute. Furthermore, they obtained a 1.26-min. decrease in total milking time as compared to 0.78 min. in this experiment. Actually, in this experiment no apparent increase in milking rate was obtained by increasing the pulsation from 50 to 80. Apparently, as the vacuum increased, the effect of an increase in pulsation rate was of progressively

less importance in increasing the rate of milking. However, 20 pulsations per minute appears below the optimum rate, even at 15 in. of vacuum.

Pulsation also affected the cows differently (shown in Table 3). The slowest-milking cows had about the same percentage increase in average rate of flow as did the four fastest-milking cows, but the slow group had a greater increase in maximum rate of flow than did the fast group as the pulsation increased.

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TECHNICAL NOTES

SOME VOLATILE COMPONENTS OF CHEDDAR CHEESE[1]

The chemical character of Cheddar cheese aroma has been under investigation at this and other laboratories for a number of years *(3, 4)*. Regarding the relative importance of any given compound or class of compounds in the aroma (e.g., fatty acids, carbonyls, amines, etc.), there seem to be substantial differences of opinion, not only between investigators but among testers. One may conclude that Cheddar aroma means different things to different people and that the total aroma is a complex mixture. Some compounds which we have identified as components of this mixture are dimethyl sulfide, ethanol, acetone, diacetyl, butanone-2, and 3-hydroxybutanone. Presumptive evidence of heptanone-2 also has been secured. With the possible exception of the latter and diacetyl, only dimethylsulfide, which was observed to be a "top-note" in all good quality Cheddar, is considered to be of obvious and direct importance to the aroma mixture.

Experiments with gas chromatography. Sections from full-cream Cheddar cheeses of good quality ranging in age from 6 mo. to a year were freed of rind and ground in a meat grinder. The ground cheese, in amounts of 1 to 5 kg., was mixed with an equal weight of distilled water. The volatiles in this mixture were removed, trapped, and analyzed by gas chromatography (GC) and mass spectrometry, using the equipment and procedures as described by Day *et al. (1)*. The distillation was carried out at from 40 to 50° C. and 30 to 40 mm. Hg pressure. Only the components recovered in dry ice–ethanol and liquid nitrogen traps were analyzed. On the basis of retention volumes by GC and mass spectral analysis of the components thus separated, dimethyl sulfide, ethanol, acetone, and diacetyl were conclusively identified. The wet-ice trap invariably had an odor suggestive of heptanone-2. A small GC peak corresponding in retention volume to that of butanone-2 also was observed. Further evidence of these two ketones in the volatiles is presented below. Several unidentified GC components with ester-like odors were noted. Although quantities of volatile components, as indicated by peak areas, appeared to vary between lots of cheese, the same components were noted rather consistently.

Experiments with volatile carbonyls: One-half kilogram quantities of ground cheese, as described above, were mixed with one liter of distilled water. This mixture was steam-distilled at atmospheric pressure. Only the first 250 ml. of distillate was collected and this was led directly from the condenser into a solution of 100 mg. of 2,4-dinitrophenyl (DNP) hydrazine dissolved in 1 ml. of concentrated H_2SO_4 and 2 ml. of water. After standing one hour, this solution was extracted with 50 ml. of benzene to recover the DNP hydrazones. The benzene extract was evaporated to a volume of 5 ml. and a 5-λ quantity of this solution paper-chromatographed by the method of Huelin *(2)*. Analysis of University cheese, as well as of six retail store samples of Cheddar, revealed DNP hydrazones falling into six well-defined groups, based on R_f values. These are shown (Table 1), together with some data on their identity. It is to be emphasized that the spots observed may have arisen from more than one compound. All the cheese samples showed evidence of most if not all of the R_f components. However, the spot at R_f 0.74 was always faint and sometimes absent. All samples showed immobile spots at the base line of chromatograms. It was assumed that these resulted at least in part from reagent and the bis-DNP hydrazone of diacetyl.

[1] Authorized for publication on February 28, 1958 as paper No. 2238 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

TABLE 1

Some properties of 2,4-dinitrophenylhydrazones from volatile carbonyls of Cheddar cheese

R_f of unknowns [a]	Knowns of corresponding R_f [b]	Absorption maximum in ethanol *(mμ)*		M.P. (° C.)		
		Known	*Unknown*	*Known*	*Unknown*	*Mixed*
0.74	Heptanone-2	362	362	58, 72, 89	.....	.....
0.61	Butanone-2	362	362	115	113	113-115
0.46	Acetone	362	361	127	.....	.....
0.29	Acetaldehyde	360	359	157, 168	.....	.....
0.19	Formaldehyde	348	350	166	.....	.....
0.06	3-OH butanone	357.5	357	115 [c]	112	112-115

[a] Paper chromatography by the method of Huelin *(2)*.

[b] Other DNP hydrazones have similar R_f's. Those derivatives listed either are considered plausible or are confirmed by data of this paper.

[c] Mono derivative.

The benzene extract of DNP hydrazones from several cheeses showing heavy spots at R_f 0.61 were pooled, evaporated to dryness, and extracted with petroleum ether (boiling, 38 to 42° C.). This extract was evaporated to dryness and the residue was recrystallized repeatedly from ethanol, yielding a maximum constant m.p. of 113° C. Admixture with an authentic sample of the butanone derivative showed no depression (Table 1).

The benzene extract of DNP hydrazones from a cheese showing an intense spot at R_f 0.06 was evaporated almost to dryness and the remaining solution streaked along the entire baseline of several chromatograms. Following development, the areas at R_f 0.06 were cut from the papers, eluted with ethanol, and the ethanol extracts evaporated nearly to dryness. Crystallization was induced by adding water. The crystals were recovered, dried, and recrystallized from a mixture of benzene and petroleum ether. This product, m.p. 112°, could not be recrystallized further, owing to limitations of quantity. It showed no depression in mixed melting point with an authentic derivative of 3-hydroxybutanone and showed essentially the same absorption maximum (Table 1).

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OBSERVATIONS ON THE DIGESTIBILITY OF ORCHARDGRASS PASTURE AS AFFECTED BY SEASON AND GRAZING MANAGEMENT[1, 2]

Recent developments *(1, 2, 4)* in research methods have made possible the determination of the grazing animal's dry matter consumption and digestibility of forages. The Western Washington Experiment Station, Puyallup, is currently using these techniques to ascertain the relative nutritive value of various grass and legume species. Some preliminary observations from this work may interest others working in this area.

Reid *(3)* previously reported a rapid decline in dry matter digestibility of first-cutting forage with advancing maturity, after a base date of April 30. Dry matter digestibility declined from approximately 77% in early May to approximately 52% by mid-July. A similar decline in digestibility with advancing maturity was reported for aftermath forage, but the initial digestibility values were not so high and the decline was not so rapid as with first growth. The digestibility of the aftermath was approximately 65% at 20 days after first cutting and declined to approximately 57.5% at 70 days.

Swift *et al. (5)* determined the digestibility of successive cuttings of orchardgrass by sheep. The forages were dried immediately after cutting. A seasonal decline was noted from spring to fall cuttings. Adding supplementary nitrogen immediately after the third cutting increased the nitrogen content of the succeeding cutting and increased the apparent digestibility of the dry matter.

The orchardgrass (*Dactylis glomerata* var. *Akaroa*) pasture used in this study was a relatively pure stand. The pasture area of approximately one acre was divided into three equal plots for rotational grazing. Three yearling Holstein heifers were put to pasture on these plots beginning April 18 and pastured continuously throughout the summer.

Grab samples of feces were collected at 6 A.M. and 4 P.M. for chromogen analyses, begin-

[1] This study was supported in part by funds from the Dairy Husbandry Research Branch under Cooperative Agreement 12-14-100-769(52).

[2] Scientific Paper No. 1693, Washington Agricultural Experiment Stations, Pullman. Project No. 1333.

ning 12 days after going on pasture. The A.M. and P.M. fecal samples were combined on an equal-weight basis for a period of seven days.

Dry matter digestibility was determined from the chromogen analyses of the feces samples, according to the method of Reid *et al. (4)*. Two digestion trials were conducted during the 1957 pasture season. The fecal collection periods during the first trial began on April 30 and continued through May 6; whereas, the second trial began on July 5 and continued through July 11. A series of five digestion trials throughout the pasture season had been planned, but technical difficulties limited the number of trials which was possible.

The dry matter digestibilities of orchardgrass by three dairy heifers during the two digestion trials are shown by days (Table 1). The individual determinations are presented to show the consistency of the daily trends between animals.

TABLE 1

Daily dry matter digestibility of orchardgrass pasture by Holstein heifers

Date	Animal No. 591	593	594	Average
	——(% digestibility)——			
	Trial 1			
April 30	73.8	72.6	72.8	73.1
May 1	73.6	72.4	72.1	72.7
May 2	71.9	71.7	70.2	71.3
May 3	71.5	71.2	69.4	70.7
May 4	69.6	70.9	68.8	69.8
May 5	69.0	69.1	67.5	68.5
May 6	66.9	68.0	66.9	67.3
		7-day	average	70.5
	Trial 2			
July 5	74.2	73.8	72.8	73.6
July 6	72.9	73.5	72.6	73.0
July 7	71.8	73.6	71.6	72.3
July 8	68.8	72.3	69.3	70.1
July 9	67.0	70.7	69.5	69.1
July 10	71.9	71.1	72.2	71.7
July 11	73.7	75.4	74.1	74.4
		7-day	average	72.0

The orchardgrass was in the early boot stage when fecal collections were started during Trial 1 and in the vegetative state during Trial 2.

Advancing maturity and grazing behavior may account for the daily variations observed in digestibility. The heifers were pastured continuously on one plot during the fecal collection period of Trial 1. The marked daily decline in digestibility during this trial partly may be a result of advancing maturity and partly due to selective grazing. Under rotational grazing the animals tend to eat the more leafy and digestible portions of the plant first when admitted to a new pasture plot. Consequently, on successive days the grazed forage becomes more stemmy and less digestible.

During Trial 2, the heifers were rotated to a new pasture plot on July 9. Therefore, the first five days of this trial represented forage that had been selectively grazed for a period before the start of the trial; whereas, the last two days represented the choice forage selected from fresh regrowth. A decline in digestibility during the first five days was followed by an increase in digestibility during the last two days. Differences in stage of maturity of the aftermath of these two plots were small at this season of the year.

These results indicate some possible errors in pasture digestibility measurements with animals on a rotational system of grazing. The fecal sampling periods should be comparable with the length of the grazing period for the entire plot. Short sampling periods, either at the beginning or toward the end of a grazing period, may not be indicative of the digestibility of the entire plot. The use of strip-grazing may be advantageous for such studies because the quality of grazed forage is more uniform.

There was no apparent decline in digestibility from early spring to aftermath forage, as reported by investigators previously. Inasmuch as orchardgrass tends toward early maturity on first growth, the forage grazed during Trial 1 may have been slightly more mature than that during Trial 2. The orchardgrass plots were maintained at a fairly high level of fertility throughout the season by the application of approximately 35 lb. of available nitrogen per acre after each grazing. Also, the plots were irrigated adequately during the grazing season.

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OUR INDUSTRY TODAY

A STUDY OF MILK DATING IN THE CITY OF NEW YORK[1]

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This study of the dating of milk in New York City was conducted during April and May, 1957. Historical and general material is limited to that necessary for a clear presentation of the results. Complete data will be available in a bulletin from the Cornell University Agricultural Experiment Station.

About 45 yr. ago, the Board of Health of the City of New York first made it mandatory, as a public health measure, to mark on the retail milk container, or bottle, the day of the week when milk was pasturized. At that time, three grades of milk could be sold in New York City, and only Grade A was suitable for children. There can be no doubt concerning the wisdom of dating milk as a public health measure in that period of the early development of a sanitary, wholesome milk supply.

Since those early days in food sanitation there have been great advances in the quality of milk and in its processing, distribution, and handling. Today the quality of milk in food stores can not be related to the day of pasteurization; consequently, it is questionable whether any public health reason remains to justify this dating regulation. Milk dealers object to this requirement as it increases the cost of milk distribution and dairy farmers suspect that it reduces sales of milk in their most profitable market. On the other hand, it is apparent that some boards of health in cities with the dating regulation are concerned with possible adverse public reactions to the removal of the regulation. At present, only 18 cities in the United States with a population over 100,000, and only two states, require dating. Last year, according to press reports, the boards of health in two of these 18 cities recommended against dating milk as a public health measure, but the common councils retained the provision. It is interesting to note that the laws of the State of New York make no provision for dating and the New York State Department of Health has opposed such a regulation within the state.

When some dairy farm organizations became concerned about this requirement in the New York City milkshed, an Assistant Commissioner in the City Department of Health pointed out that a study of the situation was necessary to ascertain the pertinent facts. At a meeting called by the New York State Commissioner of Agriculture and Markets to discuss this problem, he requested that a plan be made for such a study. Both the State Department of Agriculture and the City Department of Health offered personnel to conduct the study. Milk producers and dealers also offered to cooperate. The study was to be made immediately, to obtain enough data to show the effect of dating on milk sold through food stores. It was agreed that home deliveries and services in restaurants and hotels should not be included because dating could not be a milk-quality factor in these markets.

Areas within the city were selected on the basis of various levels of consumer income, and four types of stores were designated. Then, city sanitarians and state food inspectors were asked to select food stores of these types in the selected areas within their usual territories. These food stores were studied for one day. Also, a smaller number of food stores were studied for seven consecutive days. In adjacent municipalities outside New York City, stores were studied which handled milk marked by company code and with no time-limit on sale.

In addition to the food stores, the study included representative milk-processing plants within the City of New York, and one plant outside the city that code-marked all milk containers.

Within the city, the 187 food stores studied for one day handled 57,342 quart paper containers of milk daily, or 307 quarts per store. The wholesale deliveries were usually made before 9 A.M. and very few milk drivers rotated the dated milk in the refrigerated sales case. The 34 stores outside the city that were studied for one day handled 8,186 quarts of coded milk daily, or 241 quarts per store. Deliveries of coded milk were generally made after the store personnel arrived, and about one-third of the routemen rotated milk in the sales case.

Results with Stores Studied for One Day

The grocers' statements (and sometimes their records) showed that at least twice a month three out of four food stores had no milk for sale at closing time. The stores closed without milk an average of 1.6 times per week per store, or one-fourth of the time. One food store in eight generally had no milk for sale at closing time, and only one store in four generally had milk for sale all the time. In contrast to this prevalent lack of dated milk for sale in food stores at closing time, 67% of all grocers handling coded milk were seldom or never out of milk.

The most accurate check on the availability of milk for sale at all times was the presence or

[1] Presented at Eleventh Annual Meeting, Dairy Products Improvement Institute, Inc., February 13, 1958. New York, N. Y.

absence in the store "last night" at closing time, as reported to the survey workers. Data obtained on this basis showed that one-third of all stores handling dated milk closed business at the end of the day with no milk for sale. A Monday check revealed that 47% of all stores had been without milk when closed on Saturday or Sunday. A striking difference was found for coded milk: only one store out of 34 closed with no milk on hand.

The age of milk purchased by consumers can be ascertained most accurately and easily by an actual count of the quart paper containers offered for sale in the food stores. Milk dated today constituted 91% of the total milk on hand; 3.6% was yesterday's milk, and only 0.3% was two or three days' old, according to the date on the container. No older milk was found, even though 38,237 quarts were counted in this part of the study. An interesting aspect of this picture is that 4.5% of all milk in stores was tomorrow's milk. Predating 36 hr. is legal in the City of New York, but delivery of such milk in advance of the day marked on the container is illegal. To obtain the true age of the milk from the actual hour of pasteurization from 15 to 34 hr. must be added to the time interval.

The coded milk studied was sold outside of, but adjacent to, New York City without date and with no time-limit. Slightly over 82% of the coded milk was marked as today's milk, 15 % was yesterday's, and 2% was two or three days' old. The age of dated and coded milk as sold in food stores in New York City and in adjacent areas was the same, except for a shift of 10% from today's to yesterday's milk.

It was the custom of half of the stores to sell milk dated Saturday on Monday morning, since this time span was within the legal limits of the Sanitary Code, provided the sale was made before noon. Some stores returned some of the milk to the plants on Monday, to decrease the amount of Saturday's milk to be sold. Other stores returned all milk to the plants on Monday morning. On days not following Sundays or holidays, it was customary for three stores in four to sell yesterday's milk; that is, one store in four returned yesterday's milk to plants.

When a purchase of milk was made, the survey worker noted whether the customer examined the containers. Those who did were queried, without any leading questions, to learn why they looked at or examined the containers. All customers were classed about equally in three groups: those who did not examine containers, those who examined containers to see whether they were leaking milk, and those who looked for the date. It appears, therefore, that two-thirds of all consumers bought dated milk without looking for the date. More than half of all purchases of coded milk sold outside New York City were made without examining the container.

Results with Food Stores Studied for Seven Consecutive Days

The nine food stores handling dated milk in New York City which were studied for seven consecutive days received a total of 19,288 quart containers on regular deliveries and 1,000 quarts on special deliveries. The largest deliveries were received on Saturday and Monday. Each store closed yesterday's business with an average of 35 quarts of milk on hand, and on an average each store closed once a week with no milk for sale. Each store returned 54 quarts of yesterday's milk to the milk dealer, or 2% of all deliveries. About 90% of all milk in the stores was dated today, the remainder being equally divided between tomorrow's milk and yesterday's milk.

The four food stores handling coded milk (with no date and no time limit on sale) outside New York City, which were studied for seven consecutive days, received a total of 8,134 quarts on regular deliveries, and there were no special deliveries. The amount of milk delivered daily to each store was fairly uniform. Each of these stores closed yesterday's business with three times as much milk on hand as in the stores handling dated milk. Each store handling coded milk always had milk for sale at the close of every day's business. No coded milk was returned to processing plants for credit. About 84% of all milk in the stores was coded as today's milk; the remainder was yesterday's milk, except for 14 quarts of two-day-old milk in one store when it opened in the morning.

The dated and the coded milk were being sold within brief, comparable periods of time after pasteurization.

Studies in Milk Plants

The 11 pasteurization plants studied in New York City included the range of volume of business. They represented 31% of all plants and 28% of all milk processed for the entire city.

Packaging of milk marked with tomorrow's date began at any time between 8 A.M. and 8 P.M., according to company records. The time interval from the beginning of pasteurization to the time indicated in the Sanitary Code "for distribution after 6 A.M." of the following day varied from 10 to 22 hr. and averaged 15 hr. Actual observations by the survey workers showed this average time to be 19 hr. from pasteurization to date.

The Sanitary Code provides that the maximum interval for the date "shall not be more than thirty-six (36) hr. after pasteurization." This extra time is allowed for predating milk day-after-tomorrow to handle extra needs over holidays and weekends. The time interval from the beginning of pasteurization to date on predated milk varied from 21 to 43 hr. and averaged 34. Although predating is not universal, the plant records showed that a maximum of 50%

and an average per plant of 20%, of all processed milk was predated.

In contrast, it is well to point out that the number of hours from pasteurization to the time designated by the code for milk sold outside New York City was only 10 hr. Consequently, the age of coded milk as compared with dated milk may be slightly better than shown by the data.

Data prepared by the milk companies showed that 2% of all dated milk loaded on wholesale trucks was not sold and was returned to the plants, an amount ten times greater than for coded milk. The company data revealed that 0.6% of all dated milk sold to food stores was returned for credit, and the survey workers found the amount to be 0.7%. No coded milk (actually one quart) was returned by the retailers for credit, as found by the survey workers. These returns of dated milk were less than anticipated, but they do not include unsold today's milk (2% of the load) and unsold tomorrow's milk (3.2% of the load) returned to the plant. Together these represent 5.2% of the entire wholesale truck load of dated milk. Of the milk returned for credit from the stores to the plants, 0.41% of the total deliveries was yesterday's milk, 0.31% was two-day-old milk, and 0.06% was from three to seven days old. In view of the fact that milk beyond the three-day age was never found in the sales cases of food stores, it must be assumed that this small amount of old milk represented milk containers set aside until the truck routemen picked them up for credit.

Interviews with 85 wholesale routemen revealed that half of the deliveries of dated milk were made before the stores opened. Very few routemen rotated the milk for the grocers, even though one-fourth of them placed the milk in the sales case as instructed by the man in charge of milk at the food store. This study revealed that routemen underestimated dated milk returned to plants. Predated milk was handled on 78% of all routes and the routemen's estimates or figures showed it to be 21% of the total load, an amount in close agreement with company records.

About 4% of all processed dated milk was delivered wholesale by special deliveries, an item that must add to the cost of milk distribution. Special deliveries were a necessary part of distributing dated milk, but were held to almost nothing in the handling of coded milk.

Discussion of Study and Problem

Any discussion of sanitary milk regulations and milk quality must be predicated on the premise that safe milk of excellent quality shall be available to consumers at all times. The health department is concerned with its responsibility for safeguarding the health of its citizens, through its efforts to insure that only milk of fine quality is sold within the city. The dairy industry, particularly milk producers and milk dealers, depends for its very existence upon consumers receiving milk that is satisfactory in every respect, because consumers pleased with the quality of milk will make future purchases. The dairy industry and health departments have worked together closely in recent decades to give the citizens of this nation one of the world's finest milk supplies. The Department of Health of the City of New York, state and local regulatory agencies, educational agencies, equipment and supply companies, and the dairy industry of this milkshed have been active leaders in this progress.

The principal and only argument of consequence used to favor the dating of milk containers has been the assumption that consumers would receive milk of less age if dated and if a time limit were fixed for sale. Most persons familiar with the problem realize that the high quality of today's milk supply, together with technological and sanitation advances in the production, processing, and distribution of milk have reduced to insignificance any time supposedly gained through dating. Age of fresh milk is of much less importance than quality and handling procedures. The present study of the milk supply of New York City reduces such discussion to an absurdity, because the age of dated milk sold under a time-limit in food stores was the same as that of secretly coded milk sold with no time-limit, in neighboring municipalities.

The dating of milk is distinctly objectionable for the following reasons:

1. It places false emphasis on a date that has not affected the age of the milk and that may not reveal the age of the milk.

2. It misleads the public by associating a date with milk quality. Emphasis should be on quality only. The large majority of the cities (all but 18 over 100,000 population) and most states (all but two) do not have dated milk and there is no evidence to indicate any detrimental effect on milk quality of marketing nondated milk with no time-limit on sale.

3. It must reduce milk sales, as an appreciable percentage (34%) of food stores handling dated milk had no milk for sale at closing time. Food stores outside New York City which sold secretly coded milk almost always had milk on hand for sale.

4. The dating regulation is a needless distribution expense and irritation to the dairy industry. There is the loss owing to substantial amounts of milk being returned to plants for dumping, and the expense of special deliveries. There is the constant endeavor to exactly balance deliveries and sales in food stores, and to avoid sales of yesterday's milk to certain customers. These objectionable features of milk dating may be eliminated by coding.

Therefore, since the dating of milk containers serves little or no purpose today in sanitary milk distribution and public health control, it is recommended that dating should be deleted from the Sanitary Code. The regulations should require secret company codes with no change in the time limit from pasteurization to sale. Such a requirement will insure that no adverse change will occur in the age of the milk at any time, and that the present routine of milk deliveries will not be affected by the elimination of dating.

Some persons have expressed concern about the possibility that certain civic, social, or political leaders might arouse emotional reactions against the removal of the dating provision. In some instances, agitation by organized minority groups have produced ill will; and in others, such agitation was completely ignored by the public. I believe that any reasonable action of a board of health, taken after careful consideration of the problem, will be accepted in good spirit by an overwhelming majority of the citizens and their group leaders, because they have faith in the integrity and ability of the board members. The history of public health in this country has shown this to be true. Occasionally, criticism of active progressive leadership is voiced temporarily by minorities, and the certain approval of the majority may be delayed or not expressed. Some criticism of persons in high positions is to be expected, but it is not always forthcoming.

It has been said, too, that the public will not allow the dairy industry to save this needless expense in milk distribution, because it is not sufficient to warrant a reduction in the retail price of milk. I believe that this idea is not worthy of serious consideration in our public-spirited nation. When it is realized that the elimination of dating will not affect the age or quality of milk or the routine of distribution, but will save money in milk distribution costs and will increase sales, there will be no question about public acceptance.

An example of good acceptance of decisions made in the public interest was shown in a city that replaced dating by compulsory secret coding in 1955. In reply to my questions of January, 1957, the Senior Sanitarian in charge of milk sanitation in the city health department wrote comments which included the following statements:

"Some difficulty was experienced in selling the City Commission on coding and taking off the limited hours milk could be sold.

"One member of the City Commission worked very hard to have the amendment stopped or delayed, and especially to keep the 72-hr. limit on time the milk must be sold.

"We have had no trouble that I have heard of since the new amendment to the ordinance has been enforced. I certainly would hear of any troubles from using the dating code, if there were any competition difficulties on this coding ordinance."

NATIONWIDE RESEARCH IN CATTLE REPRODUCTION. A REVIEW

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During the past year or two, several progress reports on cattle reproduction have appeared. The review by Willett *(37)* gives a comprehensive historical account of the development of the subject. One by Roubicek *et al. (29)* is primarily intended to apply to range cattle, but much work with dairy cattle is included, and it gives a more detailed account of contemporary work than does the former paper. A general report of the work of the past 10 yr. in the northeastern states has been published *(7)*. Since these accounts are available, this review will not attempt to survey published work but will be confined to a discussion of trends and to make such suggestions as occur to the writer. Several years ago, Sykes *(31)* gave a review of the problem as it existed then, and his suggestions have formed the basis for much subsequent and valuable work, particularly in the northeastern region.

Arrangements for the exchange of information by workers in the field are well-organized. The northeastern and the western regions have Technical Advisory Committees that meet annually. These meetings enable the members and guests to review the work in progress and to make suggestions for its continuance. These committees function as part of the machinery of the Research and Marketing Act (1946) and have limited budgets for the encouragement of cooperative work on a regional basis. The northeastern project (NE 1) included work upon infective diseases as well as other aspects of the subject. It is now in its last year, but it is to be continued as two separate projects, and committees are now arranging programs, one (NE 40) dealing with pathological aspects and one (NE 41) with nonpathological ones. The western project (W2) was recently reorganized as W49. This new project emphasizes work upon the

basic nature of functional infertility. In addition, a nationwide meeting that brings together workers interested in all classes of livestock is held in alternate years. The proceedings of the first two meetings, at Iowa *(10)* and Michigan *(28)*, have been published. The 1957 meeting was held at Colorado A & M College and the proceedings of this conference also are to be published.

Almost every station has in progress one or more projects concerned in some way with bovine infertility but, in many of them, the relationship is confined to the accumulation of data incidental to the main object of the work, or with the diagnosis and treatment of such cases as arise in the station herd. They do not represent systematic work in the subject. Nevertheless, there is an increasing measure of interest in the field and, as more trained personnel become available, more serious attempts are being made to work out the basic physiology of the organs of reproduction and to improve fertility by applying the new findings.

Lifetime Efficiency

Little information on breeding efficiency can be gained from feeding and management experiments of short duration, because it takes time for most changes to make themselves felt in the reproductive tract. Where differences in feeding are involved, the reproductive organs, if they work at all, tend to work at a high degree of efficiency, especially as the developing embryo has a high priority for the available nutriment *(35)*. It draws upon the maternal system, if supply is short. Long-term experiments are necessary to uncover the effects of chronic deficiencies. Little is known of the effects of various forms of management and of feeding upon lifetime efficiency. The indications are that both may be varied within wide limits without affecting this *(7)*.

Already, there is a trend for more emphasis to be placed upon this question of lifetime reproductive efficiency. In Europe one is impressed with the high proportion of elderly cows in the barns, seemingly far more than there are in the United States. Is this due to a greater effective longevity in Europe, or is it apparent only because of a lower rate of culling for low production? Do we expect too much of our cows and wear them out too quickly? Does a high rate of production affect their reproductive efficiency? There is an increasing body of evidence that reproductive efficiency does not vary as the production level changes *(5, 8)*. But this does not necessarily mean that the cow reproduces equally efficiently at all levels of her own productive capacity. The USDA Cooperative Input-Output experiment *(19)* revealed no difference that could be attributed to feed level. This experiment lasted 2 yr. A similar experiment conducted over the lifetime of the cows would be needed to answer the question fully. Accurate records of feed and labor costs should be kept, to decide whether any prolongation of life, if it appeared, was worthwhile.

A cognate subject is the question whether the rate of growth has an effect upon longevity and upon lifetime production and reproductive efficiency. McCay *et al. (22)* showed that, in rats, slow growth is related to longer life. In this experiment the slow rate was carried to extremes; it was too slow to be applied to cattle as a practical measure. In addition, there was no measure of the value of the days of life gained. These problems are now being investigated at Cornell and at Pennsylvania, where dairy heifers and bulls have been raised at various rates of growth. The effects upon lifetime efficiency are being studied.

Nutrition and Management

In the field of nutrition, an outstanding gap in our knowledge is whether excessive fatness is a cause of sterility, either in the bull or in the cow. The question has never been put to an experimental test. Investigations abroad have suggested that reproductive failure is often due to a disturbed balance between calcium and phosphorus in the diet, especially to a wide calcium-to-phosphorus ratio *(17)*. The evidence presented in support of this view is not conclusive and the results obtained may depend on other conditions. It will be difficult and expensive to conduct a critical experiment that will settle this question, and suitable situations in which the problem may be studied in the field may be hard to find. Some pilot work with laboratory animals might help solve it.

It is most unlikely that shortages of any individual elements or compounds in the feed will cause reproductive failures, unless the supply is so short that the animal shows symptoms of the deficiency. The amounts of dry matter in the ovum and in the semen are so small that they do not represent a drain upon the available nutrients. Also, the rate of embryonic and fetal growth in cattle is so slow that the requirement for this purpose is relatively small and, as has been mentioned, the priority is high. However, it is possible that reproduction might be adversely affected by the presence of unusual substances in the feed. The existence of estrogens in certain forages is known to impair reproduction in sheep by upsetting the normal hormonic balance *(6)*. Possibly, some plants may contain substances that block the action of hormones or other substances necessary to normal function. Several instances of reduced reproduction in species other than cattle may be due to causes of this nature *(12)*.

Much more work needs to be done on the effects of management factors upon economic longevity and lifetime reproductive efficiency. Such work is necessarily time-consuming, but

it need not be expensive if it is carefully arranged. In animal husbandry research of this type, the economic implications are more important than the biological ones, so that the experiments need not be carried to extremes. Extreme experiments should first be done with laboratory animals, because biological data can usually be gathered more efficiently and faster in experiments involving small animals. Pilot tests of this kind often will help settle the limits that should be imposed in an experiment with larger animals, designed with the view of studying possible economic advantages.

Genetics

Population genetic studies have shown that, in this country, inheritance plays but a small part in the total infertility problem *(11, 26)*. The restriction must be made that this statement applies only to those heifers and cows that have shown themselves capable of conception. The rate of conception is modified by heredity in but few animals in the entire cow-population. There is still much work to be done on the impact of heredity upon absolute sterility. Any cases in which the occurrence of inherited factors is suspected should be closely analyzed, and both the nature of inheritance and of the defect should be noted. Careful analysis, too, should be made of any blood lines that are suspected of low fertility. Particular attention should be paid to the probable causes of infertility in individuals in these lines. This is a field of investigation that has scarcely been touched.

Pfau *(24)*, and other geneticists before him, has raised the question whether it is wise by treatment to preserve animals with low reproducing ability for further reproduction. He points out that a genetic gain produced by eliminating detrimental genes is permanent; whereas, preservation of such genes by enabling their possessors to reproduce may lead to the need for more treatments in subsequent generations. The argument for treatment has based itself upon the fact that only in a few instances has heredity been demonstrated, whereas an overwhelming number of conditions of relative infertility are not inherited. In the present state of our knowledge, the writer judges that the risk involved in preserving deleterious genes by treatment is small. The demonstrated inheritance rate is so low and, ironically enough, the prospects of successful treatment so slight, that the risk is minute. If a condition were found, clearly inherited but susceptible to treatment, a conflict might arise between immediate individual interest and the benefit of the breed as a whole. Most breeders would take the longer view.

Disease

At present, methods of curing animals infected with the more serious diseases of reproduction are almost entirely lacking. Brucellosis, for instance, can not be treated by any known method but, fortunately, it can be prevented almost entirely by the use of calfhood vaccination. The search continues for strains other than Strain 19 for vaccination. The metabolism of the causative organism and the immunogenic properties of its antibodies are under study at three experiment stations. More effective methods of diagnosing the disease are being sought widely and one station reports that an attempt is being made to differentiate between natural and vaccination blood titers.

Diagnosis and treatment of trichomoniasis is under study at a few experiment stations and in the Federal Agricultural Research Service. The life history of the organism is also engaging attention.

Vibriosis is attracting a good deal of attention. Several stations and the Agricultural Research Service are studying the morphology and metabolism of the *Vibrio* organism. They are developing improved methods of identification and of differentiating between pathogenic and nonpathogenic strains. At one station, diagnosis, treatment, and transmission in the bull is receiving most attention; another reports an experiment in which a usable and effective vaccine or other preventive agent is being sought and tested.

Infection with *Vibrio* is refractory to treatment in the cow, but the use of insemination, employing semen treated with antibiotics, has proved an effective method of preventing its spread. The fact that cows recover spontaneously after they have been infected is a promising feature for further investigation. What is the nature of the recovery process? Is is sufficiently permanent to confer immunity against reinfection? Could antibodies, if they have been formed, be transferred to calves?

Leptospirosis as a cause of infertility is also under investigation. Special attention is being paid to the possibility that it may be spread through the bull or by artificial insemination.

When abortions occur, it is frequently possible to rule out all the known causes. Then, why this large residue of undetected causes? So far, no one has been able to find a major cause besides those already mentioned. A few are due to leptospirosis and there is reason to suspect a virus as a further cause. Attempts to find such a virus have been tantalizing at times. Something recovered from abortuses occasionally survives in embryonated eggs for a few passages, then disappears. Whether these partial results represent the presence of a pathgenic agent is open to question. Possibly, a shift of search methods to tissue culture techniques, using endometrium or mammalian embryonic material as the medium, might yield greater success. One also wonders whether, in some instances, bacteria that are known to be individually relatively harmless may exert a

synergistic action and thus cause damage sufficient to produce abortion.

In all forms of infertility, whether or not due to known organisms, we need more information on the development of symptoms, especially on the nature of changes in the histology of the organs concerned. Perhaps endometrial biopsy may be developed to a greater usefulness when such knowledge becomes available.

As a by-product of experimental work on the effects of management and nutritional differences we may, perhaps, expect to find out whether certain practices contribute to, or limit, infection with specific organisms.

Infertility in the bull is almost a closed book. Histological examination of the testes and accessory organs of bulls of low fertility should be made. This should be a routine matter for bulls discarded from artificial-breeding studs because of low fertility. Perhaps the most vulnerable or sensitive part of the spermatogenic process or of semen production thus could be found. At least, this would provide a basis for subsequent work. One difficulty in establishing the cause of male infertility is that the epididymis is a reservoir for spermatozoa. It may take a month or more to exhaust the reservoir's supply, so that a cause of sterility may be active for some time before it is detected. A febrile condition possibly may cause temporary infertility some time after it occurs.

Artificial Insemination

Many experiment stations report work in progress on artificial insemination. Much of this work consists of a testing by each station of the newest combination of antibiotics or of diluter to be announced elsewhere. The research literature is saturated with reports of work of this nature. Not much basic work is in progress. A few stations are responsible for most of the advances that are made. Several of these are working on the possibility of preserving spermatozoa by drying in some way, and on methods of preservation and storage at room temperature. Results in the latter field, using methods of immobilizing the spermatozoa, seem to be promising *(30, 32)*. Very litle work is in progress on the metabolism of spermatozoa. Yet a knowledge of this aspect might lead to vastly improved methods of handling them.

It is difficult to visualize a function for the fluids from the accessory glands in promoting fertility, because in a normal ejaculation the spermatozoa enter them at the last moment. When the semen is deposited in the female tract, presumably the spermatozoa escape from it at once. The successful use of diluters that resemble the accessory secretions in physical properties but not in chemical composition, likewise suggests that their importance may be exaggerated. However, our knowledge of the composition of these fluids is rather limited. One does wonder whether seminal fluid from a highly fertile bull would improve the fertilizing power of semen from a bull of low fertility.

The whole question of rate of sperm travel *(33)* and of maturing factors for spermatozoa *(9, 38)* should be reopened, and current views, resting as some of them do upon single reports, checked. Would it be possible to feed a bull with radioactive material, some of which would be built into the spermatozoa? Could their radioactivity then be used to track them through the genital organs, either by the use of Geiger counters or by radio-autograph? The rate of sperm formation possibly could be estimated in the same way.

Embryonic Mortality

Embryonic mortality occasions considerable loss, and several stations are investigating the causes. It seems to be clear that good bulls fertilize a high percentage of eggs, and that losses from embryonic deaths cause most of the wastage. In bulls of low fertility, failure to fertilize as many eggs is an additional cause of failure *(4)*. When bulls of low fertility are used, treatment of the semen with antibiotics increases their nonreturn rate *(2)*, an effect observed to a much smaller extent in highly fertile bulls *(1)*. Does the addition of antibiotics increase the fertilization rate, or reduce the embryonic mortality, or both? Evidence is availible that shows a decrease in embryonic mortality *(13)*. Would the addition of specific disease factors to the semen of highly fertile bulls reduce its fertilizing power or increase the subsequent wastage? The problem is whether disease affects fertilization or the life of the embryo. If the latter, is it due to an effect upon the uterus or directly on the embryo? Probably, if semen of low fertility but without disease organisms in the fluid were used, the results of these experiments would differ.

When an embryo dies from a cause other than disease, the question arises whether the cause is in the maternal organism or in the embryo. If it is in the embryo, what causes death? Is it a failure of development in one or another of the tissue or organ systems? In order to answer these questions, detailed reference charts depicting the normal processes of development and implantation are necessary. At present these are lacking, but the gap is being filled as material becomes available *(15)*. This type of work is expensive because it is essential to obtain the material from normal cows. If it is obtained from cows culled for infertility, it may not represent the normal course of development. The expense of providing a large series of reference pictures or photographs is beyond the resources of any journal and of most experiment stations. Special provision should be made for publishing important, but expensive, bulletins of this nature. Why should not a portion of the money appropriated under the Research and Marketing Act be set aside for this purpose? Another use-

ful publication would be one giving in considerable detail the normal cyclical changes in the reproductive tract. These should be referred to ovulation time, not to the time of heat, so that a new series would be necessary to provide the material. Duration of heat and its accurate measuring are too variable for a series based on time of heat to be entirely trustworthy, as the writer has found.

More needs to be done on the problems of timing services. Most of our timing charts are related to the heat period, not to ovulation. There are some reports of surprisingly high conception rates at the extremes *(3, 34)*, and the whole question should be reopened. In this work, the exact time of ovulation should be ascertained, and the fertility percentage curve related to this as well as to the heat period. Some cases of difficulty of conception in cows may be related to ovulation at other than the modal time. Oone wonders whether spermatozoa from highly fertile bulls fertilize more eggs than those from bulls of low fertility, because they survive for a longer time in the female tract. The effect of the act of mating upon ovulation time needs reinvestigation *(21)*.

Control of Ovulation

An important step will have been taken when it becomes possible to bring the ovulation mechanism under control, so that this event can be made to take place at the will of the operator. Present indications are that, although ovulation is spontaneous in the cow, it is brought about by a secretion from the hypothalamus which travels to the anterior pituitary by way of the hypophyseal portal blood system *(16)*. The pituitary then secretes luteinizing hormone which induces rupture of the ripe ovarian follicle. The possibility of control seems to reside in the action of drugs upon the hypothalamus. But many details of the process remain to be worked out. Although the cow has the advantage over laboratory animals, that the results of treatment may be determined with a fair degree of accuracy by rectal palpation of the ovary, she is too expensive for large-scale work with little-known factors. The general control first should be worked out in smaller animals. One line of work should be directed towards finding the essential difference between the spontaneous and the induced ovulators. Another that might profitably be explored is to take advantage of the fact that some clutch-laying species of birds continue to lay if they are prevented from building up the normal clutch number. There seems to be a chain—exteroceptor, hypothalamus, anterior pituitary, ovary—which should be susceptible to detailed analysis. What causes the abrupt transition from laying (LH) to broodiness (prolactin) when the clutch is completed? When the factors involved in the chain are more certainly known, it should be easier to test them in the cow and to work out a method of controlling the process.

The theta wave of the electroencephalogram affords us an opportunity to gauge the activity of the region of the brain in and around the hypothalamus, the region of greatest immediate interest. Some study of these waves when the hypothalamus is stimulated by external factors has been made *(25)* and more use should be made of this approach. Also, when the animal is exposed to light flicker of the same frequency as the theta wave, symptoms, sometimes epileptoid, are produced that appear to be somewhat individual. Possibly, some application of this type of experiment might give us a method of sorting out bulls that would not stand up to the conditions imposed in artificial-breeding studs.

Hormone Assay

So far, all attempts to work out assay methods for sex-steroid hormones applicable to the urine or blood have been unsuccessful. This is largely due to the small amounts of these hormones that are circulating, a condition related to the great sensitivity of the bovine to these substances. It may also indicate a very rapid turnover of active steroids. Nothing is known, as yet, of their metabolism in cattle. Possibly, further applications of paper chromatography methods in this type of investigation might meet with some success. Radioactive tracer techniques are indicated to work out the rate of turnover and the metabolic pathways. The bile has been rather neglected in this respect, and it is probably a major pathway of excretion (see *27*).

Steroid chemistry is tricky; only a small change in configuration frequently makes a large difference in biological activity. The aid of one or more organic chemists with experience in both steroid chemistry and in the use of radioactive materials should be enlisted to help solve the problems outlined. We can not expect to make much progress in treating the "functional" sterilities until we are able to assay the kinds and quantities of hormones in circulation and use, so that correct balances may be restored. More accurate assays of the anterior pituitary for each of the gonadotrophic hormones, separately, in known physiological and abnormal states, are needed to indicate the secretion patterns of these hormones. Some of these suggestions await general advances in this field before applications can be made to cattle. The relationship between gonadotrophic hormone secretion and the histological picture of the anterior pituitary is also worth further study. Some progress in this direction already has been reported by Jubb and McEntee *(20)*.

Mass therapy of infertile cows with hormones has failed to yield results *(14)* but, as our knowledge of endocrine action increases, it should be possible to intervene with success in selected cases. To do so will require a deeper insight into the normal processes than we now have.

Functional Sterility

The so-called functional sterilities are still problems. Nymphomania is not understood and, at present, little or no work is being done on it although some is planned under the NE40 and 41 projects. The uterine physiology does not suggest the action of estrogens. More work upon the development of the condition, particularly as regards the ovaries and the uterus, is needed. How does the progress of the condition, when it is artificially induced by estrogen implants, resemble its natural development? What conditions predispose an animal to an attack? Is the observation *(36)* of an increase in gamma globulin in induced cases a matter of general significance? Will the incidence of the condition increase if a herd is bred along lines that assume that inheritance is involved? Can the condition be made to develop artificially in the absence of the uterus or is it a result of an endometrial overstimulation?

Persistent corpus luteum is another functional sterility that is inadequately understood. A diagnosis of this condition is made all too frequently in cases of anestrum, because ovulation without heat is more frequent than has been realized, as Hurst *(18)* has shown. The condition should not be diagnosed unless the same corpus luteum can be shown to persist as a functional body through at least two cycle-lengths. When the condition does exist, one would like to know the uterine picture as it might offer an explanation of the cause.

The occurrence of heats during pregnancy is a phenomenon that has not been explained. What is the ovarian picture when the condition occurs? In the condition of ovulation without heat, just referred to: Is this due to low sensitivity to estrogens or to their low output?

The cause of the freemartin condition is still obscure. Attempts to modify the gonads of embryos in laboratory animals by injecting the pregnant female with steroid hormones have failed *(23)*, but the method has not been tried in the bovine. Laboratory animals do not ordinarily display the freemartin condition, so that the negative results obtained when they were subjected to the treatment do not necessarily mean that the present hormone theory of its cause is faulty. There are also no reports upon the physiological and histological condition of the anterior pituitary in the freemartin.

Summary

This paper reviews the present trends in research on dairy cattle reproduction in the United States. Gaps in our knowledge are pointed out and suggestions made for work to fill them. A major need is for further work on the study of lifetime reproductive efficiency and the effects of management practices on it. The influence of heredity on the incidence of absolute sterility needs more study. In the field of infectious diseases, search should be pressed for unknown factors, especially for viruses and other subbacterial organisms. Little is known about male infertility. In artificial breeding, the role of the semen plasma needs elucidation. It is an open question whether disease affects fertilization of eggs or early embryonic mortality. Some progress is being made, at last, in mapping the early development of the calf. Timing of service for the greatest fertility should be related to ovulation time more closely than is possible at present. The problem of ovulation control, involving the neuroendocrine mechanism, should receive much more attention. Radioactive tracers should be used in the study of sex-steroid hormone metabolism. "Functional sterility" is still an obscure field that needs further investigation.

Conclusion

In the planning and interpretation of experiments with cattle, we are up against a statistical difficulty in the evaluation of the results. Surely, enough information is available to enable the statistician to tell us how many cows are needed to establish with reasonable significance whether a difference of, say, one-half service per conception between treatments is due to the treatment. Or, how many bulls, randomly chosen, are needed to give reasonable significance to a 1% difference in nonreturn rate. Such information is overdue, and it would be most useful in planning work in which the effects of various modifications of conditions are measured in terms of reproductive performance. Similar information is needed by the pathologist so that the significance of results obtained in experimental testing of therapeutic agents or methods of treatment can be gauged. In these situations, spontaneous recovery rates in animals with definite symptoms are needed for comparative purposes. Finally, what advantages over the spontaneous recovery rates are necessary to make treatment financially worthwhile at different yields?

Much of the progress in our knowledge of human physiology was obtained by careful study of abnormal conditions. These led to the recognition of the functions of the endocrine organs and to the nature of the balances necessary for their orderly function. The writer has still to see the day when an experiment station or college has the fortitude to use the better herds in its neighborhood for teaching purposes and to concentrate on collecting all the abnormal livestock it is possible to find. He is sure that an intensive study of these animals by the station staff would rapidly pay ample dividends in research, if not in public relations.

In general, it is believed that the present knowledge of reproduction and the possibility of its control in the bovine lags because many of the basic problems are still unsolved.

There is need for much more detailed work upon causes and mechanisms. Study of these

requires special skills of a high order. The writer endorses Willett's *(37)* view that men of this type should be brought in to develop those areas where the deficiency of expert knowledge is most apparent.

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OUR ASSOCIATION

GOLDEN ANNIVERSARY OF EVENTFUL MEETING AT CORNELL UNIVERSITY

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Fifty years ago, July 22 and 23, 1908, the two-year-old "Official Dairy Instructors Association" (later to become the American Dairy Science Association) held its annual meeting on the Cornell University campus, Ithaca, New York. Perhaps few meetings of the Association since then have equaled this one in the percentage rise in attendance from previous meetings, and in the number of those destined to become enshrined in the halls of dairy fame.

Membership had increased from the 17 charter members at Urbana, 1906, to 69 members from 26 states, the District of Columbia, and Canada. Forty-seven posed for a picture, which later was to become of historical interest. Eleven of those attending the third annual conference at Cornell in 1908 were to attend the 29th annual meeting in 1934, again at Cornell. At least five were present again at the 45th Cornell meeting, 1950.

Of those present at the 1908 Cornell meeting, Lee, Thom, Doane, Larson, C. W., Hunziker, Ross, Washburn, Webster, Hayden, White, Haecker, Rasmussen, Larson, C., Erf, Eckles, Pearson, Savage, Van Norman, Van Slyke, Miyawaki, Stocking, Publow, Nystrom, Dean, Guthrie, Frandsen, Armsby, Farrington, Woll, Rawl, and others, were to carve niches for themselves in dairy science and have their names revered among agricultural leaders of America during the next 50 years. Interestingly, no less than three members present, Hunziker, Eckles, and Haecker, were to be named among the all-time "Ten Master-Minds of Dairying." At least 16 were to become authors of dairy texts.

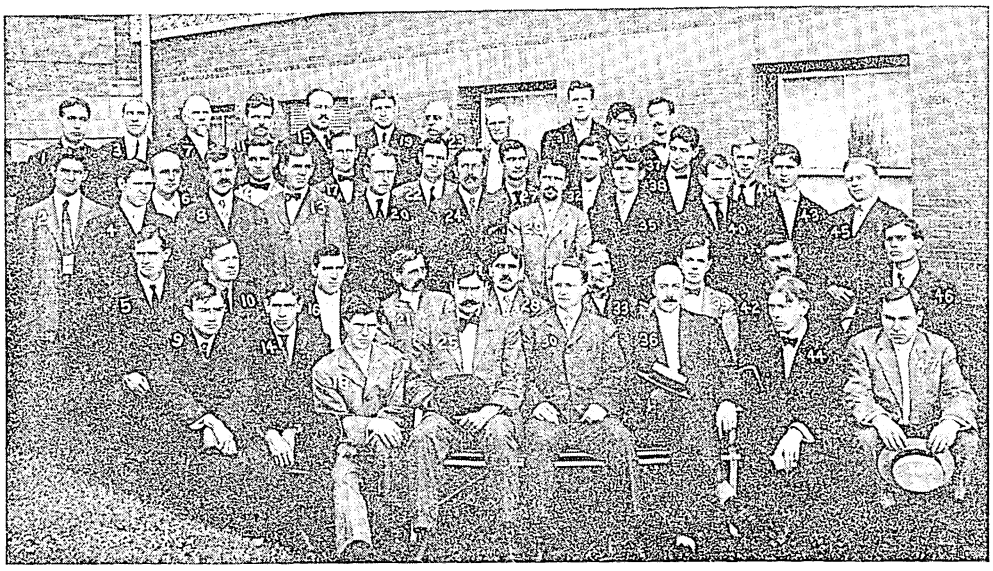
Certain characteristics destined this third conference to become of more-than-routine historical significance. In the first place, membership was up, attendance was relatively high, and interest was good. Secondly, the president, Dr. R. A. Pearson, serving his second year, confined his address to a few remarks, emphasizing the need of interesting, competent young men in dairy industry, in which good positions were awaiting incumbents. Thirdly, the program consisted not of set, topic papers by individuals, which featured the first meeting, but of thorough, thought-provoking, action-stimulating committee reports. However, the committee reports that formed the kernel of the second annual meeting at Chicago, October, 1907, must not be deprecated but, judging from the discussion of them, these reports did not carry the substance and finality of the thinking (and conclusions) of the committee members as did the (committee) reports of the Cornell conference. Fourth, these committee reports were to become, in large part, the guide for the thinking of the members of the Association for years to come. In fact, the "Report of Committee on Experimental Work: Production. Some Suggestions Regarding Methods of Conducting Experiments with Dairy Cows" by W. J. Fraser, Chairman (the founder of the Association and who was not present at the Cornell meeting), C. H. Eckles of Missouri, and H. H. Dean of Guelph laid down some basic principles in dairy cattle feeding experiments which were grasped then by the young scientists and used even to this day. With possibly one exception, full, complete reports were presented by the following committees: "Dairy score-card"; "National dairy register of merit"; "Our relations to the National Dairy Show"; "Courses of instruction"; "Experimental work: Production"; "Experimental work: Manufactures"; "Standards for dairy products"; "Extension work"; "Extension work in the United States"; "Official testing"; "Cooperative work," and "Cow test associations. "

Already, the leadership of the 2-year-old "National Association of Dairy Instructors and Investigators" was manifesting itself in the development of score-cards, standards for products, rules for dairy cattle judging contests, testing and identification of dairy cows, instruction and research, and even in dairy extension, which was not to be recognized and provided for officially by the United States Government until the passage of the Smith-Lever Act of 1914.

As a result of the Cornell conference, and through the activity of a segment of the new organization, the first college dairy cattle judging contest was set for December 3, 1908. This contest has been held almost every year since then, and has had a profound influence on the training and stimulation of future dairy leaders.

Unfortunately, the good Ithaca conference, at which so much was accomplished for the potential development of dairying, was the last of such official, summer, on-college campus meetings to be held for 19 years. However, yearly official meetings were held with the National Dairy show. On several occasions, unofficial

[1] Historian, American Dairy Science Association.



CORNELL UNIVERSITY—JULY 22ND AND 23RD, 1908

1. Lee, C. E., Urbana, Ill.; 2. Dox, A. W., Storrs, Conn.; 3. Kendall, J. C., Manhattan, Kansas; 4. Thom, C., Storrs, Conn.; 5. Ayres, H. L., Ithaca, N. Y.; 6. Willoughby, C. L., Experiment, Georgia; 7. Dexter, W. H., Washington, D. C.; 8. Doane, C. F., Washington, D. C.; 9. Campbell, P. A., Orono, Maine; 10. Larson, C. W., State College, Pa.; 11. Hunziker, O. F., Lafayette, Indiana; 12. Little, E. M., Lincoln, Nebraska; 13. Beach, C. L., Storrs, Conn.; 14. Ross, H. E., Ithaca, N. Y.; 15. Washburn, R. M., Burlington, Vt.; 16. Webster, E. H., Washington, D. C.; 17. Hayden, C. C., Urbana Ill.; 18. Ayer, L. F., Hampton, Va.; 19. White, B. D., Washington, D. C.; 20. Kent, F. L., Corvallis, Oregon; 21. Haecker, T. L., St. Anthony Park, Minn.; 22. Rasmussen, F., Durham, N. H.; 23. Whittaker, G. M., Washington, D. C.; 24. Larson, C., Brookings, Dakota; 25. Erf, O., Columbus, Ohio; 26. Merrill, L. H., Orono, Maine; 27. Jarvis, J. D., Lafayette, Ind.; 28. Eckles, C. H., Columbia, Missouri; 29. Billings, G. A., Washington, D. C.; 30. Pearson, R. A., Albany, N. Y.; 31. Savage, E. S., Ithaca, N. Y.; 32. Van Norman, H. E., State College, Pa.; 33. Van Slyke, L. L., Geneva, N. Y.; 34. Miyawaki, A., Manhattan, Kansas; 35. Melick, C. W., College Park, Maryland; 36. Lane, C. B., Washington, D. C.; 37. Stocking, W. A. Jr., Ithaca, N. Y.; 38. Caldwell, R. E., Manhattan, Kansas; 39. Publow, C. A., Ithaca, N. Y.; 40. Nystrom, A. B., Columbus, Ohio; 41. Ferguson, A., Ithaca, N. Y.; 42. Dean, H. H., Guelph, Canada; 43. Guthrie, E. S., Columbus, Ohio; 44. Simpson, O. G., Corvallis, Ore.; 45. Frandsen, J. H., Moscow, Idaho; 46. Wilson, J. K., Geneva, N. Y.; 47. Rawl, B. H., Washington, D. C.

meetings continued to be held biennially on a college campus with the summer meetings of the Graduate School in Agriculture.

Ironically, the National Association of Dairy Instructors and Investigators, founded so well at Urbana in 1906, and relaunched with full steam at Cornell in 1908, was due for at least "Seven Lean Years," if not longer. A "dark era" in the life of the 2-year-old association was ahead. During this period, little about the Association seems to have been recorded. The founding of the Journal in 1917 gave the Association new life. The re-establishment of the summer on-college campus meetings at East Lansing in 1927 was the beginning of an upsurge in attendance at the annual meetings of the Association.

The influence of the annual meetings of the American Dairy Science Association such as the one at Cornell, 1908, can be measured only in the lives it has touched. And these are immeasurable. Radiating out from distinguished men such as were gathered at this eventful meeting, were forces destined to leave their imprint on the minds of those within their range. Only after a life of active service may these influences be viewed in proper perspective. Reading between the lines of Amer B. Nystrom's recollections of the 1908 Cornell meeting, after half a century, one gets a hint of the satisfaction and sustaining force which can be had by attending the meetings of the American Dairy Science Association. He writes:

I was a youngster—only 1 year out of college, when I went to Ithaca for the 1908 sessions and I felt very fortunate to be able to attend. Aside from the business sessions, which I don't remember at all, I took in all the lectures and demonstrations on dairying, bacteriology, and general subjects that did not conflict. I particularly remember Dr. W. W. Stocking's talks on Dairy Bacteriology. Also B. D. White's on Market Milk and C. F.

Doane's on Cheesemaking. Dr. Van Slyke also talked on Cheesemaking.

Prof. Oscar Erf, then at Ohio State, was one of the leaders in the Dairy Instructors Association's sessions at that meeting. The photo mentioned above shows him and other officers in the front row, as I recall. I can't remember who the other officers were, but you may be able to figure it out from the picture.

I don't believe Dr. S. M. Babcock was there, but we had demonstrations and talks on the Babcock test, which, as I recall, were given by either Farrington or Woll or both. Then we had a couple of lectures on nutrition by Dr. Armsby. He talked about the energy (calorie) value of feeds and their effects on dairy cow health and production.

Outside of the dairy field, I remember some excellent general talks by Dean Liberty Hyde Bailey which I enjoyed. And one evening Dean Bailey had us all in for a social evening at his home. There he talked to us again, in an entertaining way which we all appreciated.

Perhaps the stimulus engendered by attending the annual Association meetings, as reflected in the above letter, can not be evaluated in its entirety. In time, the attendance at the annual meeting becomes an urge for the dairy scientist. Many of those attending the third annual meeting on the Cornell campus, 1908, welcomed the opportunity to return again and again to the annual gathering. They learned early the meaning of President W. A. Stocking, Jr.'s words spoken at Columbus, Ohio October 14, 1917, "It is a pleasant thing for us to get together each year, and throw off the restraint when we have a chance, and visit informally."